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Señor Superintendente
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E. S. D.

Trámite : 011
Evento : 378
Actuación : 412

Referencia : Expediente No. **13.132.887**
Asunto : Solicitud de patente para: **"UNA COMPOSICIÓN FARMACÉUTICA PARA UN SUJETO QUE TIENE MÉTODO PARA TRATAR DIARREA ASOCIADA CON VIH O DIARREA ASOCIADA CON TERAPIA ANTIRETROVIRAL ALTAMENTE ACTIVA (HAART)"**
Solicitante : **SALIX PHARMACEUTICALS, LTD. y NAPO PHARMACEUTICALS, INC.**
Fecha de solicitud : 30 de Mayo de 2013

I. RECURSO VÍA GUBERNATIVA

Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad **SALIX PHARMACEUTICALS, LTD. y NAPO PHARMACEUTICALS, INC.** solicitante de la patente de la referencia, por el presente escrito atentamente manifiesto a usted que debidamente notificada de la Resolución No. **645** del 15 de Enero de 2015, interpongo en su contra el recurso de **REPOSICIÓN**, para que sea revocada y en consecuencia se conceda el privilegio de patente de invención para **"UNA COMPOSICIÓN FARMACÉUTICA PARA UN SUJETO QUE TIENE MÉTODO PARA TRATAR DIARREA ASOCIADA CON VIH O DIARREA ASOCIADA CON TERAPIA ANTIRETROVIRAL ALTAMENTE ACTIVA (HAART)"** a favor de **SALIX PHARMACEUTICALS, LTD. y NAPO PHARMACEUTICALS, INC.**

II. OPORTUNIDAD DE LA IMPUGNACIÓN

La Resolución No. **645** del 15 de Enero de 2015, con fecha de publicación listado del 20 de Enero de 2015, fecha de vencimiento del 20 de Febrero de 2015, y fecha del término legal el 6 de Marzo de 2015.

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

III. LA DECISIÓN RECURRIDA

- 3.1 Mediante la Resolución objeto de la presente impugnación, el Superintendente de Industria y Comercio decidió:
- 3.1.1 Denegar la patente de invención a la creación titulada: **“UNA COMPOSICIÓN FARMACÉUTICA PARA UN SUJETO QUE TIENE MÉTODO PARA TRATAR DIARREA ASOCIADA CON VIH O DIARREA ASOCIADA CON TERAPIA ANTIRETROVIRAL ALTAMENTE ACTIVA (HAART)”**
- 3.2 En la parte motiva de la providencia, se mencionaron los siguientes fundamentos:
- 3.2.1 “Que la solicitante mediante escrito radicado bajo el N° 13-132887-00009-0000 del 01 de octubre de 2014, respondió oportunamente el requerimiento formulado nueva(s) reivindicación(es) 1 a 9 que reemplaza(n) las originalmente presentadas, atendiendo de esta manera las observaciones efectuadas en el examen de fondo. Sin embargo, no se acepta el nuevo capítulo reivindicatorio presentado, comoquiera que no se ajusta a las prescripciones contenidas en el artículo 34 de la Decisión 486, porque aunque las nuevas reivindicaciones 1-9 se encabecen como composición, lo reclamado es claramente un método de tratamiento ya que dichas reivindicaciones mencionan las diferentes dosis y la raza de los individuos en los cuales se ensayó el CROFELEMER (polímero de proantocianidina, CAS 148465-45-6), lo que se evidencia en los ejemplos de la descripción.”
- 3.2.2 “Por otro lado, se observa que en la descripción no se encuentra ningún ejemplo específico mediante el cual se haya desarrollado una composición, y por el contrario, de acuerdo con dicho capítulo descriptivo, el problema técnico no se plantea ni se divulga en términos del diseño y desarrollo de una composición farmacéutica verdadera, ya que sólo se menciona un listado de formas farmacéuticas y excipientes farmacéuticamente aceptables que podrían ser incluidos en las formas farmacéuticas de las citadas, lo cual no es sustento de una composición farmacéutica, por lo tanto no hay divulgación suficiente para cambiar el objeto de método de tratamiento a composición. De manera que la divulgación gira en torno a estudios clínicos, con individuos VIH positivos que padecen de diarrea y sus síntomas, para determinar las dosis efectivas de

crofelemer en dicho método de tratamiento, ver por ejemplo (tablas 9-11, pág. 77-82). Por las mismas razones tampoco fue aceptado el capítulo reivindicatorio del radicado No 13-132887-00006 -0000. En consecuencia, se tendrá en cuenta el capítulo reivindicatorio presentado con el radicado No 13-132887-00000 -0000.”

3.2.3 “La(s) reivindicación(es) 1 a 33, no se acepta(n) por cuanto está(n) referida(s) a: “Un método para tratar diarrea asociada con VIH o diarrea asociada con terapia antiretroviral altamente efectiva (HAART) en un sujeto VIH positivo, que comprende: administrar aproximadamente...), el cual no puede ser objeto de patente de acuerdo al artículo citado.”

3.2.4 “Sin perjuicio de lo anterior ya existían documentos publicados con fecha anterior a la fecha de presentación de la solicitud (citados por la sociedad opositora) de la cual se invoca prioridad, que revelaban el compuesto CROFELEMER y composiciones que lo contienen: D1, D2, D3 y D4.”

3.2.5 “Es oportuno aclarar que, el capítulo presentado con la respuesta no es aceptado, porque a pesar de que las reivindicaciones se encabezan por composición, la materia reclamada corresponde a un método de tratamiento, como se explicó claramente en el considerando cuarto, por lo tanto esta Oficina considera pertinente reiterar que:

- Encabezar una reivindicación en términos de composición no es suficiente para concluir que existe sustento en cuanto al diseño y desarrollo de una composición verdadera.
- Una composición siempre se define en términos de ingrediente activo, excipientes, diluyentes y sus proporciones. La presencia del núcleo que soporta el ingrediente activo no puede ser opcional cuando hay un verdadero diseño y desarrollo de un sistema de entrega farmacéutico.
- De acuerdo con el capítulo descriptivo el problema técnico no se plantea ni se divulga en términos del diseño y desarrollo de una composición farmacéutica verdadera, por lo tanto no hay divulgación suficiente para cambiar el objeto de método de tratamiento a composición. Toda la divulgación gira en torno a la determinación de dosis efectivas del compuesto CROFELEMER (polímero de proantocianidina, CAS 148465-45-6).

- El hecho de establecer una dosis efectiva para un tratamiento específico es un elemento propio del método de tratamiento, en este caso del método para tratar en el tratamiento de sujetos VIH positivos para controlar síntomas como la diarrea entre otros.”

3.2.6 “De otro lado, esta Oficina no sugirió otro título y el propuesto ahora, por el solicitante, a pesar de estar encabezado por una composición también se refiere a un método de tratamiento.”

3.2.7 “Asimismo, la solicitante afirma que “los documentos D1/D2 y D4 se encuentran dirigidos a métodos de tratamiento de sujetos que tienen el síndrome del intestino irritable con diarrea como síntoma predominante (SII-D), que es una condición clínica distinta de la diarrea asociada a VIH y de la diarrea asociada a HAART, tal como lo caracteriza las reivindicaciones pendientes... D3 se encuentra dirigido a la evaluación de crofelemer para el tratamiento del Síndrome del Intestino Irritable con estreñimiento como síntoma predominante (SII-C). Basado en las enseñanzas de los documentos D1 a D4, no habría sido evidente para una persona del oficio normalmente versada en la materia que las composiciones farmacéuticas reivindicadas para sujetos que tienen diarrea asociada a VIH o diarrea asociada a HAART que comprende una dosis de crofelemer de menos de aproximadamente 1.000 mg podía ser utilizado para los sujetos que tienen diarrea asociada al VIH o diarrea asociada a HAART, ya que diferentes tipos de diarrea tienen diferentes causas fisiológicas, y un régimen de tratamiento que es eficaz para un tipo de diarrea puede no ser eficaz en el tratamiento de otros tipos de diarrea.”

3.2.8 “Contonea afirmando que: “Un experto en la técnica entendería que, simplemente porque un fármaco es eficaz para el tratamiento de un tipo de diarrea no significa que el mismo fármaco será eficaz para el tratamiento de otros tipos de diarrea, especialmente si los tipos de diarrea resultan de diferentes causas fisiológicas”.

3.2.9 “A los anteriores argumentos se aclara que la condición distinta como diarrea asociada a VIH y diarrea asociada a HAART, no es una característica de una composición. Por otro lado, en cuanto a la siguiente afirmación: “diferentes tipos de diarrea tienen diferentes causas fisiológicas, y un régimen de tratamiento que es eficaz para un tipo de diarrea puede no ser eficaz en el tratamiento de otros tipos de diarrea”, se aclara que, el hecho de utilizar el CROFELEMER (polímero

de proantocianidina, CAS 148465-45-6) para un régimen de tratamiento dirigido a otra condición de salud, no es una característica técnica de una composición y por el contrario está confirmando que lo soportado en la descripción es un régimen de tratamiento el cual no es materia patentable dentro de la norma vigente (Decisión 486), como se explicó antes.”

- 3.2.10 “Asimismo, a la afirmación de que “...simplemente porque un fármaco es eficaz para el tratamiento de un tipo de diarrea no significa que el mismo fármaco será eficaz para el tratamiento de otros tipos de diarrea, especialmente si los tipos de diarrea resultan de diferentes causas fisiológicas”, esta Oficina observa, que con esta afirmación la solicitante muestra que la invención no consiste en una composición sino en un método de tratamiento.”
- 3.2.11 “También asegura que “contrario a la afirmación del señor Examinador y a la afirmación de la Sociedad Opositora, una persona del oficio normalmente versada en la materia no habría estado motivada a modificar las composiciones farmacéuticas de los documentos D1 a D4 que se utilizan para tratar sujetos que tienen SII-D o SII-C, para llegar a las composiciones farmacéuticas reivindicadas, las cuales comprenden menos de 1,000 mg de crofelemer para un sujeto que tiene diarrea asociada con VIH o HAART”.”
- 3.2.12 “A lo anteriormente argumentado, esta Oficina aclara que no existe soporte de composición alguna con la cantidad de 1000mg, y que por el contrario de acuerdo a lo soportado lo que se evidencia es el uso de diferentes dosis desde 125 hasta 1000mg, la cual si fuera del caso, la dosis de 1000mg, se puede obtener con las composiciones divulgadas en el arte previo citado como se aclaró antes.”
- 3.2.13 “Por todo lo anterior, si fuera del caso analizar, se reitera que los documentos D1 a D4 ya revelaban composiciones que comprenden el compuesto CROFELEMER (polímero de proantocianidina, CAS 148465-45-6); por lo tanto, no se está pasando por alto ninguna diferencia con respecto al estado de la técnica ya que no existe tal diferencia, y por lo tanto tampoco existe nivel inventivo.”

IV. OBJETO DEL RECURSO

De acuerdo con los argumentos que expongo a continuación, solicito atentamente a su Despacho lo siguiente:

1. Revocar la Resolución No. **645** del 15 de Enero de 2015.
2. Conceder el privilegio de patente de invención para **“UNA COMPOSICIÓN FARMACÉUTICA PARA UN SUJETO QUE TIENE MÉTODO PARA TRATAR DIARREA ASOCIADA CON VIH O DIARREA ASOCIADA CON TERAPIA ANTIRETROVIRAL ALTAMENTE ACTIVA (HAART)”** a favor de **SALIX PHARMACEUTICALS, LTD.** y **NAPO PHARMACEUTICALS, INC.**

V. PRESENTACIÓN DE UNA SOLICITUD DIVISIONAL Y MODIFICACIÓN DE UNA SOLICITUD EN TRÁMITE

En virtud del Artículo 36 de la Decisión 486 mediante el cual se establece la posibilidad de dividir la solicitud en dos o más fraccionarias, se presenta en esta oportunidad una solicitud divisional, junto con un pliego reivindicatorio conformado por 33 reivindicaciones, donde en la solicitud parental se encuentran las reivindicaciones relacionadas con composiciones farmacéuticas que comprende como ingrediente activo crofelemer y en la solicitud divisional las reivindicaciones relacionadas con un método para tratar diarrea asociada con VIH o diarrea asociada con terapia antiretroviral altamente activa (HAART) en un sujeto VIH positivo.

Teniendo en cuenta que se cumplen con los requerimientos exigidos por el artículo mencionado, se solicita atentamente dar trámite a esta solicitud divisional.

Por otra parte, de acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 9 reivindicaciones.

A este respecto, el señor Examinador tomó la decisión de no aceptar el pliego reivindicatorio presentado el 01 de octubre 2014 junto con la respuesta a la Acción Oficial No. 5991 porque supuestamente "a pesar de que las nuevas reivindicaciones 1 a 9 se refieren a una composición, la materia reivindicada es claramente un método de tratamiento." En concreto, el señor Examinador ha indicado que el pliego reivindicatorio de la solicitud presentada anteriormente no se encuentra soportado en la descripción, porque

"no hay suficiente divulgación para cambiar el objeto del método de tratamiento a composición."

En vista de dichas objeciones, respetuosamente manifestamos nuestro desacuerdo y nuevamente presentamos adjunto con este documento el mismo pliego reivindicatorio presentado el 01 de octubre 2014 junto con la respuesta a la Acción Oficial No. 5991. A este respecto, amablemente señalamos que el nuevo pliego reivindicatorio se encuentra debidamente soportado en la descripción. De acuerdo a la memoria descriptiva, tal como fue presentada ante su Despacho, en la página 28, líneas 5 a 7 y en la página 30, líneas 4 a 23 se encuentra el debido soporte para las reivindicaciones presentadas junto con este recurso y si bien el pliego reivindicatorio originalmente presentado se refería a métodos de tratamiento, las composiciones objeto de protección en el nuevo pliego reivindicatorio se encuentran igualmente soportadas en la descripción tal como fue originalmente presentada y por lo tanto, la objeción formulada a este respecto se debe tener por superada.

Adicionalmente, la GUÍA PARA EXAMEN DE SOLICITUDES DE PATENTE DE INVENCION Y MODELO DE UTILIDAD de la Superintendencia de Industria y Comercio establece lo siguiente:

"Cuando la descripción menciona además de los métodos de tratamiento, los elementos generales que conforman las composiciones tales como los excipientes y principios activos, aunque no describa una composición específica, entonces el examinador aceptará la modificación del capítulo reivindicatorio de método de tratamiento a composición."

De acuerdo a lo anterior, es claro que cuando las reivindicaciones se encuentran dirigidas a excepciones a la patentabilidad, es decir, a métodos de tratamiento, las mismas pueden ser modificadas para que definan la composición de interés, siempre y cuando, dicha composición se encuentre debidamente soportada en la descripción. De esta forma, tal como ya se demostró y sustentó anteriormente, la composición reivindicada se encuentra clara y enteramente soportada en la descripción en las páginas página 28, líneas 5 a 7 y en la página 30, líneas 4 a 23.

En consecuencia, solicitamos el estudio y aceptación de las reivindicaciones modificadas presentadas con este recurso.

Por lo tanto, los argumentos esgrimidos a continuación con respecto a la patentabilidad de la invención, en los términos del capítulo reivindicatorio modificado, resultan plenamente predicables del capítulo reivindicatorio sobre el cual la patente fue negada. En efecto, la no ampliación de la materia en el nuevo pliego es evidencia suficiente de que el presente recurso controvierte el análisis de patentabilidad efectuado por su Despacho, de tal manera que el presente recurso cumple con los requisitos de ley para su aceptación y trámite.

VI. FUNDAMENTOS DEL RECURSO

Respetuosamente me permito disentir de lo expuesto por ese Despacho en la Resolución objeto del presente recurso, toda vez que la presente solicitud sí cumple con los requerimientos de novedad y nivel inventivo exigidos en la Decisión 486, como se demostrará a continuación:

6.1 La presente solicitud sí tiene novedad y nivel inventivo

El artículo 16 de la Decisión 486 señala lo siguiente:

“Artículo 16.- Una invención es nueva cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya sido accesible al público, por una descripción escrita u oral, por una utilización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.”

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis de novedad de una solicitud de patente, se considera que cuando las características técnicas esenciales son idénticas a las características de la materia divulgada en un documento del estado de la técnica, puede afectar dicho requerimiento de patentabilidad, en cuyo caso se considera que no tiene novedad. Adicionalmente, se considerará nueva la invención si se encuentra alguna diferencia o un sólo aspecto que no hubiese sido revelado en el documento del arte previo.

Adicionalmente, el artículo 18 de la Decisión 486 señala lo siguiente:

“Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.”

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

Respecto a lo anterior, el Tribunal de Justicia de la Comunidad Andina ha expuesto en la Interpretación Prejudicial del proceso 13-IP-2010:

“En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado”.

[señalado fuera del texto]

Por lo anterior, se entiende que para que una persona medianamente versada en la materia pudiera derivar de manera obvia una invención, requeriría simplemente aplicar sus conocimientos técnicos. En contraste, todo aquello que va más allá de la simple aplicación de los conocimientos técnicos y que no ha sido revelado en el estado de la técnica, debe ser considerado inventivo.

Con base en lo anterior, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta la novedad y el nivel inventivo, con base en la siguiente información:

En primer lugar ponemos de presente que debido a que los documentos D1 y D2 son documentos de patente homólogos de PCT y Estados Unidos, éstos serán abordados en conjunto en la siguiente discusión. Así las cosas, los documentos D1/D2 y D4 se encuentran dirigidos a métodos para tratar ***el síndrome del intestino irritable con diarrea como síntoma predominante (SII-D)***, en donde una persona del oficio versada en la materia sabría que es una condición clínica distinta de la diarrea asociada a VIH y a la diarrea asociada a HAART. De manera similar, el documento D3 se encuentra dirigido a la evaluación de crofelemer para el tratamiento del ***Síndrome del Intestino Irritable con estreñimiento como síntoma predominante (SII-C)***.

Como nota preliminar, se encuentra bien documentado que las enfermedades diarreicas debido a diversas causas mostrarán uno o más mecanismos discretamente diferentes. Los cuatro tipos principales de diarrea son: diarrea osmótica; diarrea secretora; diarrea asociada con anomalías de la motilidad; y la diarrea exudativa. Aunque las causas precisas de la diarrea asociada al VIH son actualmente desconocidas, la diarrea en pacientes con VIH es una diarrea exudativa que resulta de la alteración de la función epitelial de barrera por la liberación de citoquinas estimulada por el VIH y, con infección avanzada por VIH, diarrea que se asocia con malabsorción y diarrea osmótica. Así las cosas, en vista de los argumentos 6 y 11 de la Declaración de Herbert L. Dupont del 15 de agosto 2013 (Anexo C), no existe una correlación reconocida en la técnica entre el tipo de diarrea en d-IBS frente a la diarrea asociada al VIH y los procesos involucrados son "tan completamente diferentes que no sería obvio que el crofelemer es eficaz en el tratamiento de la diarrea asociada a VIH y la diarrea asociada a HAART a dosis baja reivindicada, solo porque el crofelemer es eficaz en el tratamiento de la diarrea asociada con d-IBS en una dosis baja" (párrafo 12 de la Declaración del 15 de agosto 2013).

Basado en las enseñanzas de los documentos D1 a D4, no habría sido evidente para una persona del oficio normalmente versada en la materia que las composiciones farmacéuticas reivindicadas para el uso en el tratamiento de diarrea asociada a VIH o diarrea asociada a HAART que comprende una dosis de crofelemer de menos de aproximadamente 1.000 mg podía ser utilizado en el tratamiento de diarrea asociada al

VIH o diarrea asociada a HAART, ya que ***diferentes tipos de diarrea tienen diferentes acusas fisiológicas, y un régimen de tratamiento que es eficaz para un tipo de diarrea puede no ser eficaz en el tratamiento de otros tipos de diarrea.***

Un experto en la técnica entendería que, simplemente porque un fármaco es eficaz para el tratamiento de un tipo de diarrea no significa que el mismo fármaco será eficaz para el tratamiento de otros tipos de diarrea, especialmente si los tipos de diarrea resultan de diferentes causas fisiológicas.

Hay varios ejemplos en la literatura científica en la que un fármaco es eficaz para un tipo de diarrea, pero no es eficaz para el tratamiento de otros tipos de diarrea. Un ejemplo es Sandostatine, un análogo de la somatostatina de acción prolongada, que ha demostrado ser útil para el tratamiento de la diarrea asociada al VIH. Sin embargo, también se ha demostrado que el uso de Sandostatin en pacientes con síndrome del intestino irritable con diarrea como síntoma predominante (SII-D y no SII-c) “no tiene un efecto analgésico visceral y no mejora los síntomas del síndrome del intestino irritable”. Un ejemplo de lo anterior, se demuestra en el artículo Conclusions de Klooker *et al.*, (2007), Effect of long-term treatment with octreotide on rectal sensitivity in patients with non-constipated irritable bowel syndrome, *Alimentary Pharmacology & Therapeutics*, Vol. 26: 605-615.

Otro ejemplo es Loperamida (también conocido como Imodium). Aunque se ha encontrado que la Loperamida es eficaz para el tratamiento de la diarrea secretora, se ha encontrado que los pacientes con diarrea asociada al VIH tienen una mala respuesta a la Loperamida. Lo anterior se encuentra demostrado en el primer párraf, columna derecha, página 166 de Katabira, (1999), Epidemiology and management of diarrheal disease in HIV – infected patients, *Int. J. Infect. Dis.*, Vol. 3(3): 164-167.

Otro ejemplo es Atapulgita, que ha demostrado ser eficaz para el tratamiento de la diarrea secretora, pero se encontró que era ineficaz en el tratamiento de la diarrea asociada al VIH. Dado lo anterior, un ejemplo se puede encontrar en el artículo, Nwachukwu and Okebe, Antimotility agents for chronic diarrhea in people with HIV/AIDS, *Cochrane Database Syst. Rev.*, October 8, 2008: CD005644.

Por lo tanto, contrario a la afirmación del señor Examinador y a la afirmación de la Sociedad Opositora, una persona del oficio normalmente versada en la materia no habría

estado motivada a modificar las composiciones farmacéuticas de los documentos D1 a D4 que se utilizan para tratar sujetos que tienen **SII-D o SII-C**, para llegar a las composiciones farmacéuticas reivindicadas, las cuales comprenden menos de 1,000 mg de crofelemer para un sujeto que tiene **diarrea asociada con VIH o HAART**.

Por otra parte, los solicitantes respetuosamente señalan que los mecanismos de acción exactos de diarrea en sujetos no siempre se conocen, y **no hay evidencia de que el tipo de diarrea asociada con SII-D (según lo enseñado por D1/D2 y D4) y SII-C (según lo enseñado por D3) y el tipo de la diarrea asociada con el VIH y HAART (tal como se reivindica en la presente solicitud) son el resultado de mecanismos de acción comunes**, por lo tanto, una persona versada en la materia no esperaría que una composición farmacéutica que es eficaz para tratar SII-D y SII-C sería eficaz para un sujeto que tiene un tipo diferente de diarrea, y en particular, diarrea asociada con VIH y HAART.

En vista de lo anterior, los Solicitantes respetuosamente indican que en el momento de la presentación de la presente solicitud, no había ninguna indicación, enseñanza o sugerencia de que las composiciones farmacéuticas reivindicadas que comprenden una baja dosis de crofelemer también serían composiciones farmacéuticas eficaces en la misma baja dosis para sujetos que tienen diarrea asociada a VIH o diarrea asociada a HAART, tal como se establece en las reivindicaciones pendientes de la presente solicitud.

De esta forma, se ven superadas las objeciones con respecto a novedad para la presente invención toda vez que es claro que los documentos D1 a D4 no anticipan como un todo las características técnicas esenciales del método objeto de protección tal como se encuentra reivindicado en el nuevo pliego reivindicatorio.

De acuerdo con los argumentos anteriormente explicados, la invención de la presente solicitud de patente es novedosa sobre los documentos del estado de la técnica D1, D2, D3 y D4 citados por el Examinador.

Por otra parte, un técnico con conocimientos medios en la materia no estaba en condiciones de plantearse el problema, tampoco de resolverlo en la forma en que se

reivindica y tampoco de prever el resultado, por lo que la presente solicitud posee la altura inventiva para que se le conceda el privilegio solicitado.

En consecuencia de lo anterior, la invención de la presente invención tiene nivel inventivo en vista de los documentos D1, D2, D3 y D4 del estado de la técnica citados por el Examinador.

6.2 Con respecto a la garantía del debido proceso:

Ahora bien, el Examinador en la resolución en cuestión indica que la materia reclamada en el pliego reivindicatorio modificado en respuesta a la anterior acción oficial No. 5991 amplía el alcance de la descripción inicial y por lo tanto, reitera las mismas objeciones formuladas en dicha acción oficial.

A este respecto, respetuosamente manifestamos al Despacho, que no podemos compartir la decisión de denegar la patente reiterando las mismas objeciones formuladas en la acción oficial NO. 5991 al no aceptar el pliego reivindicatorio modificado propuesto, toda vez que se opone al texto y al espíritu del Artículo 45 de la Decisión 486 así como por violar la garantía del debido proceso consagrada en el artículo 29 de la Constitución Política.

Lo anterior, por cuanto el Examinador insiste en que el pliego reivindicatorio modificado amplía el alcance de la solicitud inicial más sin embargo, no desvirtúa los argumentos que demostraban el sustento descriptivo de la composición objeto de protección en la presente solicitud y presentados durante el trámite de la solicitud y adicionalmente el análisis de patentabilidad incluido en la resolución impugnada **frente a las enseñanzas del documento D1, D2, D3 y D4,** corresponde aquel previamente efectuado por el Examinador en la Acción Oficial No. 5991 y no se tuvieron en cuenta las modificaciones efectuada y/o los argumentos esgrimidos por mi representada en respuesta a la acción oficial mencionada con los cuales se demostraba la patentabilidad de la composición recitada en el nuevo pliego reivindicatorio. Así entonces si se parte entonces del texto del artículo previamente citado, en el presente caso su Despacho solamente estaba facultado para negar la patente en los siguientes casos:

(i) si el solicitante no hubiera dado respuesta al Oficio **No.5991**, o

(ii) si la respuesta a este no hubiera sido suficiente para subsanar los impedimentos notificados.

No obstante, y como es afirmado en la primera página de la resolución impugnada, mi representada no solo dio respuesta oportuna al Oficio **No. 5991**, mediante escrito radicado el día 1 de octubre de 2014, sino que en dicha respuesta se siguieron TODAS las recomendaciones que sobre claridad efectuó el examinador y se demostró que la materia objeto de protección en el nuevo pliego reivindicatorio, contrario a las afirmaciones del Examinador, SI se encontraba debidamente soportada en el pliego descriptivo tal como fue originalmente presentado.

Así entonces, a partir del texto del artículo 45 de la Decisión 486 lo que resultaba forzoso para ese Despacho, era otorgar la patente solicitada, por cuanto TODAS las objeciones trasladadas habían sido superadas con la respuesta dada por mi representada y en la resolución pendiente el Examinador no menciona expresamente las razones por las cuales no considera que los argumentos presentados no demuestran el soporte de las composiciones objeto de protección en el pliego descriptivo original sino que insiste en los argumentos previamente mencionados en la acción oficial NO. 5991 sin tener en cuenta los argumentos y modificaciones presentados por mi presentada en respuesta a dicho oficio y con los cuales se consideraba por superada las objeciones formuladas.

Adicionalmente, la decisión de negar la patente con base en las objeciones previamente formuladas por el Examinador en la acción oficial NO. 5991 y decidir negar la presente invención sin referirse a los argumentos presentados en respuesta a dicho oficio con los cuales se consideraba por superada la objeción de nivel inventivo, viola el espíritu del artículo 45 de la Decisión 486 que pretende, como toda la legislación internacional sobre esta misma materia, discutir con el solicitante las objeciones existentes, de tal manera **que solo se adopte una decisión final una vez oído aquel.**

Sin embargo, en el presente caso a mi representada se viola su derecho a ser escuchada en descargos frente a las objeciones que sobre la solicitud de patente puede tener su Despacho.

Esta garantía que sirve de fundamento al artículo 45 de la Decisión 486, tiene un fundamento constitucional, en el derecho al debido proceso que debe tener toda persona en Colombia, es decir, al derecho de que se respeten las formalidades propias de toda actuación judicial y administrativa, como en el presente caso. De esta manera, al

adoptarse una decisión que no esté conforme con el procedimiento propio del trámite de patentes, no solo se conculca el derecho del solicitante a la patente solicitada sino al debido proceso.

Por las anteriores razones, respetuosamente debemos apartarnos de la decisión adoptada por su Despacho en la resolución impugnada, toda vez que claramente la misma se encuentra motivada indebidamente y se aplica una regla jurídica que no se encuentra consagrada en nuestro ordenamiento jurídico.

Conclusión

El documento D1, el documento D2, el documento D3, el documento D4, o su combinación no resulta anterioridad válida capaz de afectar la novedad y el nivel inventivo de la invención de la presente solicitud.

En consecuencia, el mérito investigativo de la presente invención ha sido reconocido por otras oficinas de patentes del mundo, las cuales han otorgado patentes para invenciones equivalentes. Tal es el caso de Estados Unidos de América, patente No. US8,962,680.

A este respecto, deseamos poner de presente que si bien cada país es autónomo de conceder o negar una solicitud luego de la realización del estudio de fondo, es claro que el hecho de que la novedad y el nivel inventivo de una invención hayan sido reconocidos por varias oficinas a nivel mundial, es un indicio de la patentabilidad de la misma toda vez que (i) el estudio de novedad y nivel inventivo en todas las Oficinas de Patentes del mundo es realizado por personas con conocimientos técnicos en el área de interés que son consideradas personas idóneas para emitir conceptos sobre la patentabilidad de una invención y que (ii) los resultados de los exámenes de novedad o de patentabilidad efectuados respecto a solicitudes extranjeras pueden ser reconocidos como suficientes para acreditar el cumplimiento de las condiciones de patentabilidad de la invención, tal como se señala en el artículo 46 de la Decisión 486:

“Artículo 46.- La oficina nacional competente podrá requerir el informe de expertos o de organismos científicos o tecnológicos que se consideren idóneos, para que emitan opinión sobre la patentabilidad de la invención. Asimismo, cuando lo estime conveniente, podrá requerir informes de otras oficinas de propiedad industrial.

De ser necesario, a efectos del examen de patentabilidad y a requerimiento de la oficina nacional competente, el solicitante proporcionará, en un plazo que no excederá de 3 meses, uno o más de los siguientes documentos relativos a una o más de las solicitudes extranjeras referidas total o parcialmente a la misma invención que se examina:

a) copia de la solicitud extranjera;

b) copia de los resultados de exámenes de novedad o de patentabilidad efectuados respecto a esa solicitud extranjera;

(...)

*La oficina nacional competente podrá **reconocer los resultados de los exámenes referidos en el literal b) como suficientes para acreditar el cumplimiento de las condiciones de patentabilidad de la invención*** (Negrilla fuera del texto).

Por todo lo anterior, consideramos que la presente solicitud cumple a cabalidad con los requerimientos de patentabilidad exigidos por la legislación actual, por lo que atentamente solicitamos en consideración lo siguiente:

VII. PETICIÓN

Por todo lo anterior, solicito a ese Despacho lo siguiente:

7.1 Que al considerar las razones precedentes se revoque la decisión contenida en la Resolución No. **645** del 15 de Enero de 2015, en cuanto resuelve negar el privilegio de patente de invención.

7.2. Que se acepte el nuevo pliego reivindicatorio presentado con este escrito, conformado por 9 reivindicaciones.

7.3. Que se otorgue el privilegio de patente de invención para **“UNA COMPOSICIÓN FARMACÉUTICA PARA UN SUJETO QUE TIENE MÉTODO PARA TRATAR DIARREA ASOCIADA CON VIH O DIARREA ASOCIADA CON TERAPIA ANTIRETROVIRAL ALTAMENTE ACTIVA (HAART)”** a favor de **SALIX PHARMACEUTICALS, LTD.** y **NAPO PHARMACEUTICALS, INC.**

VIII. NOMBRE Y DIRECCIÓN

El nombre y la dirección del solicitante recurrente son los siguientes:

SALIX PHARMACEUTICALS, LTD.

8510 Colonnade Center Dr., Raleigh,
Carolina del Norte 27615,
Estados Unidos de América

y

NAPO PHARMACEUTICALS, INC.

185 Berry Street, Suite 1300, San Francisco,
California 94107,
Estados Unidos de América

IX. ANEXOS

- Formulario de modificaciones en solicitud pendiente
- Recibo por concepto de presentación de modificaciones a las reivindicaciones por un valor de \$132.000
- Nuevo pliego reivindicatorio conformado por 9 reivindicaciones
- Solicitud de división de una solicitud en trámite
- Memoria descriptiva de la solicitud divisional
- Pliego reivindicatorio divisional conformado por 33 reivindicaciones
- Comprobante de tasa por la solicitud divisional por valor de \$478.000
- Comprobante de pago por 23 reivindicaciones adicionales después de la décima por valor de \$ 736.000 para la solicitud divisional
- Copia del Poder
- Copia de la Patente de Estados Unidos de América, junto con sus reivindicaciones modificadas
- Declaraciones de Helbert L. Dupont
- Anexo A: Hoja de Vida de Dupont
- Anexo B: Shah, 2004
- Anexo C: Field, 2003
- Anexo D: Stockmann, 1997
- Anexo E: Fanning, 1991
- Anexo F: Klooker, 2007

- Anexo G: Katabira, 1999
- Anexo H: Nwachukwu, 2008
- Anexo I: Morris, 1999
- Anexo J: Sharp, 2010
- Anexo K: Hecht, 1999
- Anexo L: Tomson, 2005

X. NOTIFICACIONES

Atentamente manifiesto a usted que recibiré notificaciones en la Secretaria de su Despacho o en mi oficina de abogada situada en la Carrera 4ª. No. 72-35, piso 4º, de esta ciudad de Bogotá, D.C.

Señor Superintendente,


LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-20242-0841-001-5
SGD\SGD\ID-278660\WPC20242
No. M-2015-278660\SGD

 Industria y Comercio SUPERINTENDENCIA	Espacio reservado para el adhesivo de radicación
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DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Trámite

☐ Error material

☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural

☐

Persona Jurídica

☒

SALIX PHARMACEUTICALS, LTD.

Y

NAPO PHARMACEUTICALS, INC.

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América	8510 Colonnade Center, Dr., Raleigh, Carolina del Norte 27615, Estados Unidos de América	Raleigh
Y	Y	y
Estados Unidos de América	185 Berry Street, Suite 1300, San Francisco California 94107, Estados Unidos de América	San Francisco
Respectivamente	Respectivamente	Respectivamente
Dirección electrónica		No. Fax
clientes@cavelier.com		2 11 86 50
		Número telefónico
		3 47 36 11

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3.

Datos del apoderado:

Apellidos y Nombre

De Páez Luz Clemencia

Documento de identificación

C.C. 35.456.344

Tarjeta profesional

T.P.A. 23.555

Dirección y domicilio

Carrera 4ª No. 72 –35

Ciudad

Bogotá

Dirección electrónica

cavelier@cavelier.com

No. Fax

2 11 86 50

Número telefónico

3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4.

Datos del trámite

Corrección:

Expediente No

13.132.887

Aparece

Debe ser

Modificación:

☒

Modificación al capítulo reivindicatorio.

Presento nuevo capítulo reivindicatorio compuesto por 9 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas. Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y además se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

☐

Modificación al capítulo descriptivo.

☐

Modificación al resumen.

☐

Modificación a los dibujos.

5.

Anexos

☒

Comprobante de pago de la tasa para la presentación de la corrección y/o modificación por \$132.000

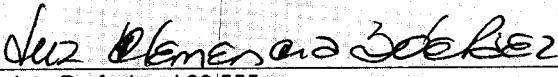
☐

Poder, si fuere el caso.

☒

Documento que contiene las modificaciones o correcciones.

PI02-F17 vr1 (2012-04-19)

6. Firma	
Nombre y apellidos LUZ CLEMENCIA DE PAEZ	Firma 
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

SGD\SGD\NCA\ID-AF278675\WPC20242

 Industria y Comercio SUPERINTENDENCIA	Espacio reservado para el adhesivo de radicación
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DIRECCIÓN DE NUEVAS CREACIONES

CONVERSIÓN, DIVISIÓN Y FUSIÓN DE SOLICITUDES

1. Identificación del Trámite

☐ Conversión

☒ División

☐ Fusión

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural☐

Persona Jurídica☒

SALIX PHARMACEUTICALS, LTD.

y

NAPO PHARMACEUTICALS, INC.

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa

Micro☐

Pequeña☐

Mediana☐

Otra☐

Documento de identificación:

☐ C.C.

☐ C.E.

☐ NIT

☐ Otro Número

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América	8510 Colonnade Center, Dr., Raleigh, Carolina del Norte 27615, Estados Unidos de América	Raleigh
Y	Y	y
Estados Unidos de América	185 Berry Street, Suite 1300, San Francisco California 94107, Estados Unidos de América	San Francisco
Respectivamente	Respectivamente	Respectivamente
Dirección electrónica	No. Fax	Número telefónico
clientes@cavelier.com	2 11 86 50	3 47 36 11

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

EB
CB

3. Datos del apoderado:

Apellidos y Nombre

De Páez Luz Clemencia

Documento de identificación

C.C. 35.456.344

Tarjeta profesional

T.P.A. 23.555

Dirección y domicilio

Carrera 4ª No. 72 –35

Ciudad

Bogotá

Dirección electrónica

cavelier@cavelier.com

No. Fax

2 11 86 50

Número telefónico

3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite

CONVERSION

Expediente

De:

A:

DIVISION

Expediente : 13.132.887

De:

A:

FUSION

Expediente

De:

A:

5.

Recibo No 15-0025520

Fecha: 2015/03/03

Recibo No 15-0026366

Fecha: 2015/03/05

6. Anexos

☒ Comprobante de pago de la tasa de presentación solicitud por \$478.000

☒ Comprobante de pago por 23 reivindicaciones adicionales después de la décima reivindicación por \$736.000

☒ Poder, si fuere el caso con el que se acredita la representación

☒ Documento que contiene la conversión, división o fusión de la solicitud

☐ Copia de la solicitud y sus anexos en formato magnético

7. Firma

Nombre y apellidos

LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 DE Usaquén

Firma

Luz Clemencia de Páez

Tarjeta Profesional 23.555

SGD\SGD\NCA\ID-AF278678\WPC20242

PI02-F05 vr6 (2012-04-19)

NOTA: Esta es una solicitud de división de la materia contenida originalmente en la solicitud de patente No. 13.132.887. La solicitud de división de la materia se hace como consecuencia de la decisión proferida por ese Despacho por medio de la resolución No. 645 del 15 de enero de 2015. Por lo tanto pido que esta solicitud de división de materia sea tratada con el beneficio de presentación de la solicitud original, esto 31 de octubre de 2011 y las prioridades que allí se reivindican el 31 de octubre de 2010, 02 de Noviembre de 2010 y 22 de Noviembre de 2010.

Nota: La copia básica original se encuentra en el expediente No. 13.132.887

REIVINDICACIONES

1. Una composición farmacéutica para un sujeto que tiene diarrea asociada con VIH o diarrea asociada con terapia antiretroviral altamente activa (HAART), que comprende:

de 125 mg a 1000 mg de crofelemer;

un portador farmacéuticamente aceptable seleccionado de azúcares, lactosa, glucosa y sacarosa, almidones, almidón de maíz y almidón de papa, celulosa, y sus derivados, celulosa microcristalina, carboximetilcelulosa de sodio, croscarmelosa, etilcelulosa y acetato de celulosa, tragacanto pulverizado, malta, gelatina y talco;

un excipiente seleccionado de celulosa microcristalina, manteca de cacao y ceras para supositorios, aceites, aceite de cacahuete, aceite de semilla de algodón, aceite de cártamo, aceite de ajonjolí, aceite de oliva, aceite de maíz y aceite de soya, glicoles, propilenglicol, siliconas, dióxido de silicio, ácido silícico, talco, polioles, glicerina, sorbitol, manitol y polietilenglicol, ésteres, estearato de magnesio, oleato de etilo y laureato de etilo, agar, agentes reguladores, hidróxido de magnesio e hidróxido de aluminio, ácido algínico, agua libre de pirógenos, solución salina isotónica, solución de Ringer, alcohol etílico, y soluciones reguladoras de fosfato.

2. La composición farmacéutica de la reivindicación 1, en donde el sujeto es de origen Caucásico o Hispano.

3. La composición farmacéutica de la reivindicación 1, en donde el sujeto es una mujer.

4. La composición farmacéutica de la reivindicación 1, caracterizada porque comprende aproximadamente 125 mg de crofelemer.

5. La composición farmacéutica de la reivindicación 1, caracterizada porque comprende aproximadamente 250 mg de crofelemer.

6. La composición farmacéutica de la reivindicación 1, caracterizada porque comprende aproximadamente 500 mg de crofelemer.

7. La composición farmacéutica de la reivindicación 1, caracterizada porque comprende aproximadamente 1000 mg de crofelemer.

8. La composición farmacéutica de la reivindicación 1, en donde el portador farmacéuticamente aceptable se selecciona del grupo que consiste de celulosa o uno de sus derivados, celulosa microcristalina, carboximetilcelulosa de sodio, croscarmelosa, etilcelulosa, acetato de celulosa, tragacanto pulverizado, malta, gelatina y talco; siliconas, dióxido de silicio, ácido silícico y estearato de magnesio.

9. La composición farmacéutica de la reivindicación 1, en donde los excipientes se seleccionan del grupo que consiste de celulosa microcristalina, manteca de cacao, ceras para supositorios, aceites, aceite de cacahuete, aceite de semilla de algodón, aceite de cártamo, aceite de ajonjolí, aceite de oliva, aceite de maíz y aceite de soya, glicoles, propilenglicol, siliconas, dióxido de silicio, ácido silícico, talco, polioles, glicerina, sorbitol, manitol, polietilenglicol, ésteres y estearato de magnesio.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

-/-

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RECIBO DE CAJA

No. 15 - 0026369

Bogotá D.C., Marzo 05 de 2015 - 11:21:32

RECIBIDO DE : CAVELIER ABOGADOS

NI 860.041.367

RE

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
RECIBO DE CA	999999999	25506	03/03/2015		109.000.00
RECIBO DE CA	999999999	25513	03/03/2015		435.000.00
RECIBO DE CA	999999999	25514	03/03/2015		435.000.00
RECIBO DE CA	999999999	25518	03/03/2015		286.000.00

*** Conceptos Pagados ***

CANT. RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1 50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES EN CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA		132.000.00	132.000.00
			\$132.000.00

SON: **CIENTO TREINTA Y DOS MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-132887-00016-0000

Fecha: 2015-03-05 16:24:20 Dep. 2020 DIR.NUEVASCR
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Act. 412 REPOSPRESRECU Folios: 247

0010-20242-841-001-5

Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia

Web: www.sic.gov.co e-mai: info@sic.gov.co Conmutador: (571) 5870000 Fax: (571) 5870284 Línea: 018000-910165 Call Center: (571) 6513240

, Marzo 5 de 2015 - 11:21:33

25
26



(12) **United States Patent**
Forbes et al.

(10) **Patent No.:** **US 8,962,680 B2**
(45) **Date of Patent:** ***Feb. 24, 2015**

(54) **METHODS AND COMPOSITIONS FOR
TREATING HIV-ASSOCIATED DIARRHEA**

(75) Inventors: **William Forbes**, Raleigh, NC (US);
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None
See application file for complete search history.

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(57) **ABSTRACT**

Presented herein are methods for treating diarrhea by admin-
istering to a patient in need thereof, an inhibitor of chloride-
ion transport in an amount sufficient to treat diarrhea. Treat-
ment of diarrhea includes the treatment of the diarrhea as well
as the pain, abdominal discomfort and other symptoms asso-
ciated with diarrhea. In one embodiment, the inhibitor of
chloride-ion transport is crofelemer.

27 Claims, 3 Drawing Sheets

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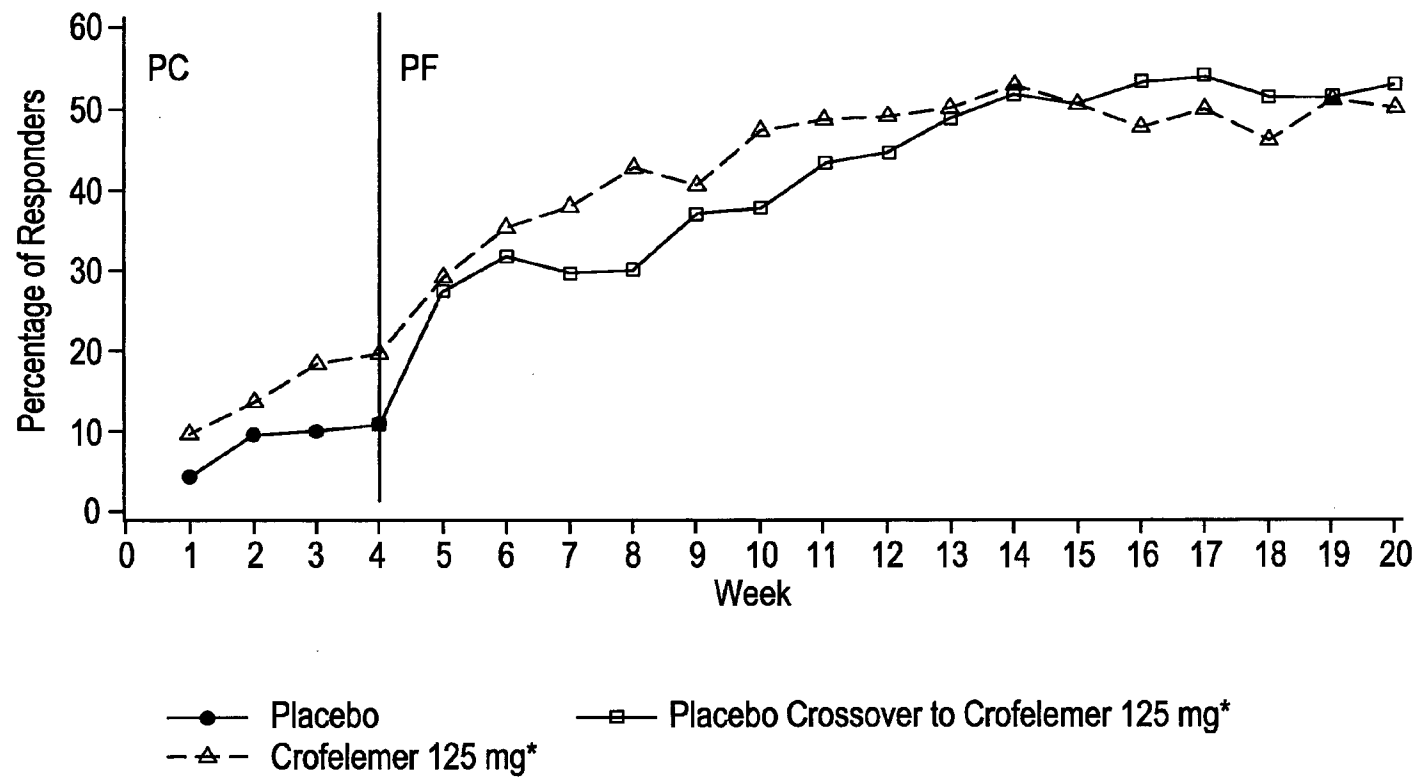
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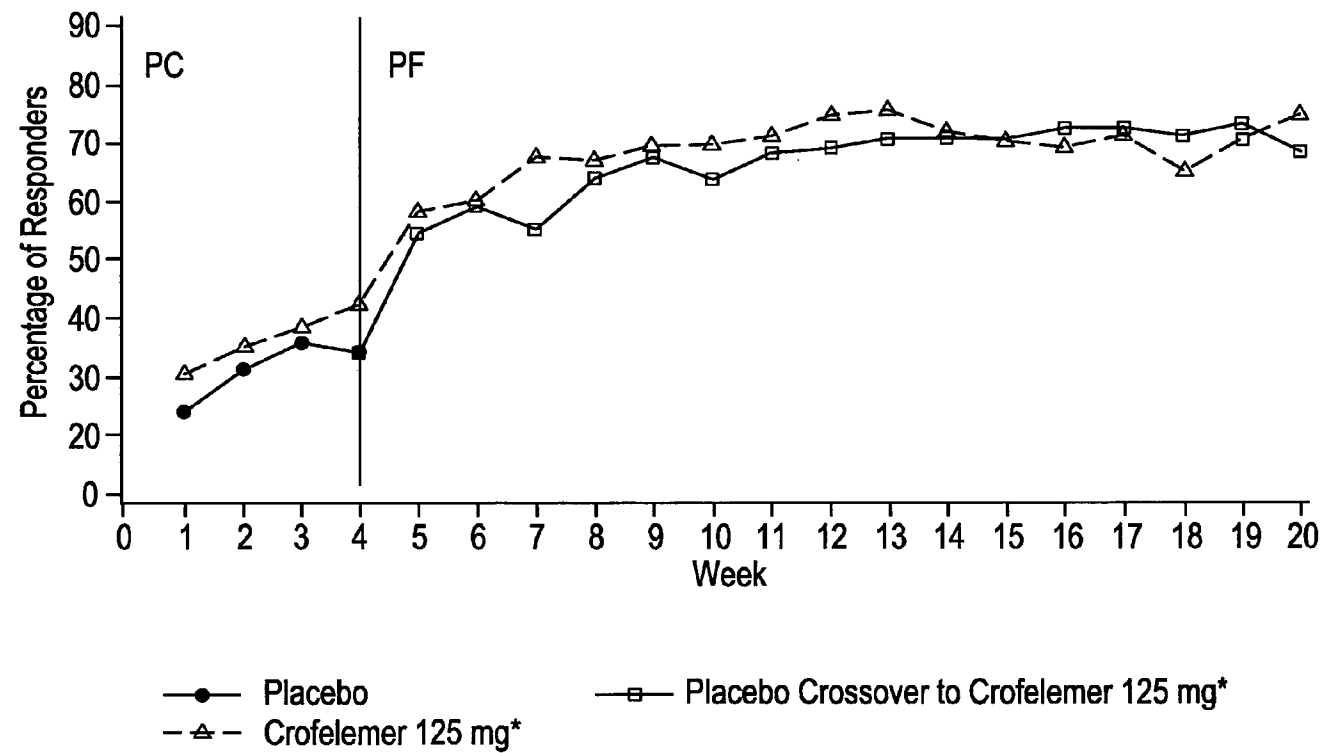
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FIG. 1



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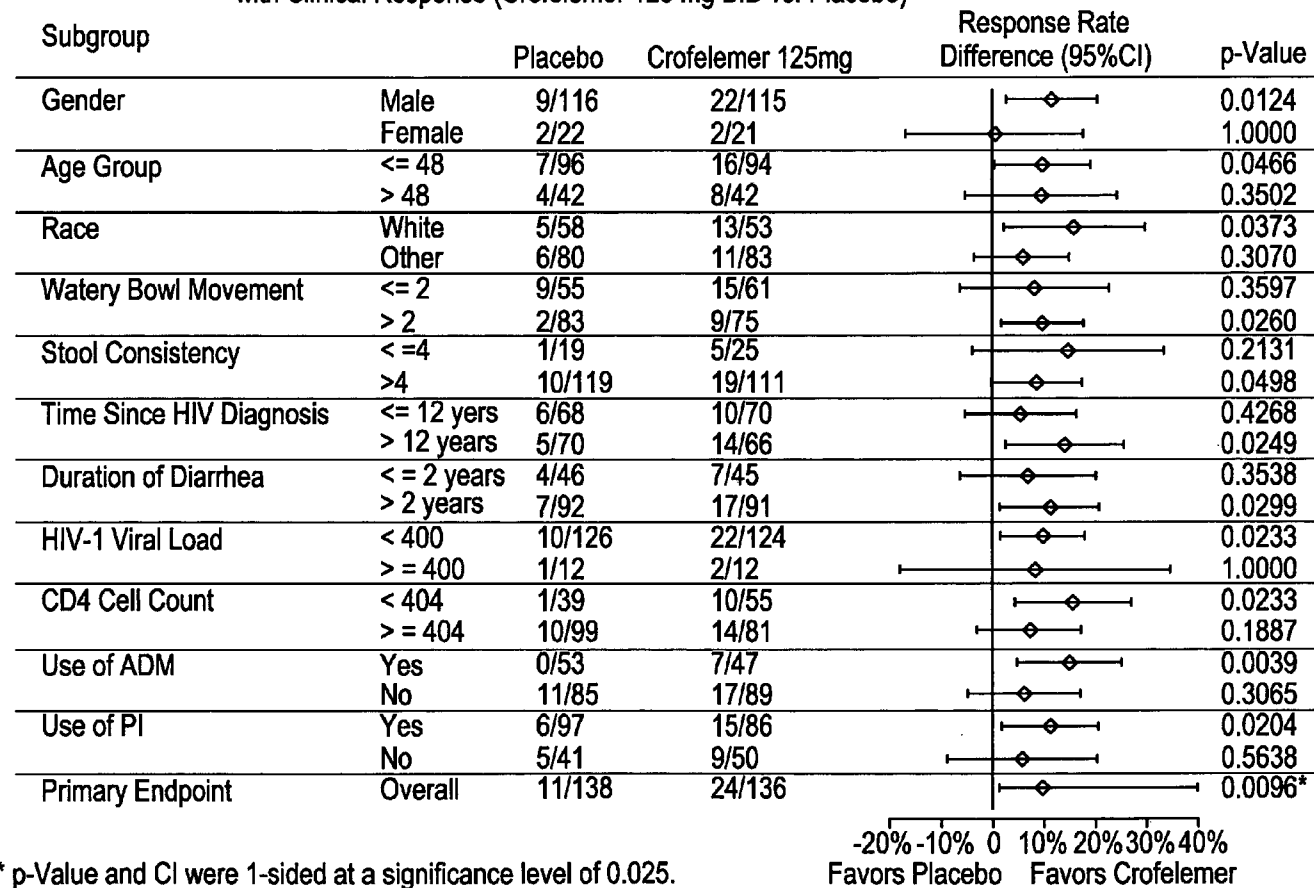
FIG. 2



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FIG. 3

Subgroup Analyses -Primary Endpoint: Percent of Subjects
with Clinical Response (Crofelemer 125 mg BID vs. Placebo)



* p-Value and CI were 1-sided at a significance level of 0.025.

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METHODS AND COMPOSITIONS FOR TREATING HIV-ASSOCIATED DIARRHEA

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/408,622, filed Oct. 31, 2010; U.S. Provisional Application No. 61/409,335, filed Nov. 2, 2010; U.S. Provisional Application No. 61/416,249, filed Nov. 22, 2010; and U.S. Provisional Application No. 61/434,379, filed Jan. 19, 2011. The entire contents of each of these applications is hereby incorporated in herein by reference.

BACKGROUND

Diarrhea remains an important problem for HIV-infected subjects in the highly active antiretroviral therapy (HAART) era, impacting negatively on quality of life, despite the extensive use of anti-diarrheals. Causes are many and include HIV enteropathy, overgrowth of unusual microbial agents, common enteric pathogens malignancy, and adverse effects of HAART therapy itself (Kartalija 1999).

While definitions and methods of reporting vary, it is estimated that around half of all HIV-AIDS subjects will have diarrhea at some point during their illness. Although the incidence of diarrhea did not change during the introduction of HAART, the etiologies of diarrhea changed significantly with an increase of noninfectious causes and a decrease in opportunistic infectious causes.

Managing diarrhea will assist in improving overall efficacy of anti-viral drug therapies, as well as quality of life, and controlling weight loss in HIV-positive subjects. Diarrhea may result in reduced antiretroviral compliance and/or necessitate switching ARV regimens. Diarrhea has also been associated with reduced antiretroviral drug levels, suggesting that adequate treatment will improve the absorption of ARV medication. On a population-wide basis, adherence to drug treatment regimens and maintenance of adequate ARV levels are important for minimizing the development of drug resistant strains of the virus. Therefore, drug-related diarrhea in HIV-positive subjects represents an important and unmet clinical need requiring more effective management. Currently prescribed therapies are only partially effective or are plagued by unacceptable side effects such as constipation and the potential for addiction. The development of a drug for the treatment of HIV-associated diarrhea with a low potential for drug-drug interactions, effects on drug metabolism, or abuse potential would provide an important benefit for HIV-infected subjects.

SUMMARY

Disclosed herein are methods of preventing, ameliorating and/or treating diarrhea. In one embodiment, the methods presented herein prevent, ameliorate or treat HIV-associated diarrhea and highly active antiretroviral therapy (HAART) associated diarrhea.

In one aspect, presented herein are methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, presented herein are methods of treating stool consistency in an HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, presented herein are methods of improving stool consistency in an HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, presented herein are methods of alleviating watery diarrhea in an HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, presented herein are methods of decreasing the number of bowel movements per day in an HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one embodiment, the administering is from between about 1 month and about 5 months. In one embodiment, the administering is from between about 1 month and about 6 months. In one embodiment, the administering comprises about 6 months. In another embodiment, the administering comprises 6 months or longer.

In one embodiment, the administering is from between about 3 days and 5 months. In one embodiment, the administering is from between about 3 days and 6 months.

In one embodiment, improvement of symptoms begins on day 3.

In one embodiment, improvement of symptoms increases with a longer duration of administration after day 3.

In one embodiment, the subject is of Caucasian or Hispanic descent.

In one embodiment, the crofelemer is administered for at least 8 days.

In one embodiment, the crofelemer is administered from between 8 days and 24 weeks or 8 days and 26 weeks.

In one embodiment, a subject is considered treated if the subject demonstrates one or more of a decrease in the number of bowel movements per day, a decrease in the number of watery bowel movements per day, an improvement in the daily abdominal score for pain or discomfort, an improvement in the score for daily stool consistency, a decrease in stool consistency score (from watery to formed), a decrease in the number of days per week that subjects experienced urgency, a decrease in the number of days per week that subjects experienced fecal incontinence, or a decrease in the unscheduled visit for a significant worsening of diarrhea.

In one embodiment, a subject is considered treated if the subject demonstrates an improvement in the score for daily stool consistency.

In one embodiment, a subject is considered treated if the subject demonstrates a decrease in stool consistency.

In one embodiment, a subject is considered treated if the subject demonstrates a decrease in the number of watery bowel movements per day.

In one embodiment, subject is considered treated if the subject demonstrates a decrease in the number of bowel movements per day.

In one embodiment, symptoms increased or decreased are measured from a baseline.

In one embodiment, the administering is for about 5 months. In another embodiment, the administering is for about 6 months.

In one embodiment, the administering is about 5 months or longer. In another embodiment, the administering is about 6 months or longer.

In one embodiment, the administering is for the duration of the HIV infection.

In one embodiment, the response to treatment increases after the crofelemer has been administered for longer than 4 months.

Other embodiments are disclosed infra.

DESCRIPTION OF THE FIGURES

FIG. 1 shows subjects with clinical response in the crossover to placebo-free phase of the safety population.

FIG. 2 shows subjects with stool consistency response in the crossover to placebo-free phase in the safety population.

FIG. 3 depicts a subgroup analysis showing the treatment difference in percentage of responders (crofelemer 125 mg BID vs. placebo) with associated confidence intervals and p-values.

DETAILED DESCRIPTION

The methods disclosed herein involved the administration of effective amounts of a proanthocyanidin polymer, e.g., crofelemer, to subjects having, for example, HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea.

Proanthocyanidins are a group of condensed tannins. Crude extracts from medicinal plants, for example, *Pycnanthus angolensis* and *Baphia nitida*, have been shown to have antidiarrheal qualities in animal tests (Onwukaeme and Anuforo, 1993, *Discovery and Innovation*, 5:317; Onwukaeme and Lot, 1991, *Phytotherapy Res.*, 5:254). Crude extracts which contain tannins, in particular extracts from carob pods and sweet chestnut wood, have been proposed as treatments or prophylactics (U.S. Pat. No. 5,043,160; European Patent No. 481,396).

Proanthocyanidins are comprised of at least two or more monomer units that may be of the same or different monomeric structure. The monomer units (generally termed "leucoanthocyanidin") are generally monomeric flavonoids which include catechins, epicatechins, gallo catechins, galloepicatechins, flavanols, flavonols, and flavan-3,4-diols, leucocyanidins and anthocyanidins. Therefore, the polymer chains are based on different structural units, which create a wide variation of polymeric proanthocyanidins and a large number of possible isomers (Hemingway et al., 1982, *J. C. S. Perkin*, 1:1217). Larger polymers of the flavonoid 3-ol units are predominant in most plants, and are found with average molecular weights above 2,000 daltons, containing 6 or more units (Newman et al., 1987, *Mag. Res. Chem.*, 25:118).

Proanthocyanidin polymers are found in a wide variety of plants, particularly those with a woody habit of growth (e.g.,

Croton spp. and *Calophyllum* spp.). A number of different *Croton* tree species, including *Croton sakutaris*, *Croton gossypifolius*, *Croton palanostima*, *Croton lechleri*, *Croton erythrochilus* and *Croton draconoides*, found in South America, produce a red viscous latex sap called Sangre de Drago or "Dragon's Blood". U.S. Pat. No. 5,211,944 first described the isolation of an aqueous soluble proanthocyanidin polymer composition from *Croton* spp. and the use of the composition as an antiviral agent (See also Ubillas et al., 1994, *Phytomedicine*, 1:77). The proanthocyanidin polymer composition was shown to have antiviral activity against a variety of viruses including, respiratory syncytial, influenza, parainfluenza and herpes viruses. U.S. Pat. No. 5,211,944 also discloses the isolation of an aqueous soluble proanthocyanidin polymer composition from *Calophyllum inophyllum* and the use of this composition as an antiviral agent.

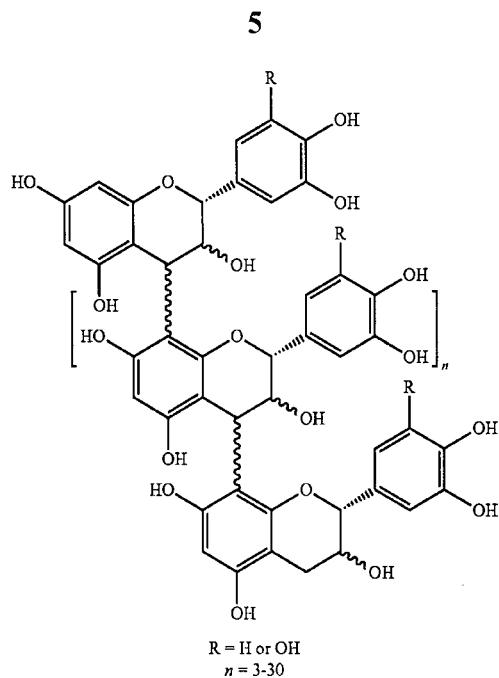
Exemplary proanthocyanidin polymer compositions useful in the methods presented herein are preferably isolated from a *Croton* spp. or *Calophyllum* spp. by any method known in the art. For example, the proanthocyanidin polymer composition may be isolated from a *Croton* spp. or *Calophyllum* spp. by the method disclosed in U.S. Pat. No. 5,211,944 or in Ubillas et al., 1994, *Phytomedicine* 1: 77-106.

Proanthocyanidin polymer compositions useful in the methods presented herein may also be made in vitro using synthetic techniques.

In one specific embodiment, a proanthocyanidin polymer composition useful in the methods presented herein is crofelemer.

Crofelemer is an oligomeric proanthocyanidin extracted and purified from the red, viscous latex of the plant *Croton lechleri* of the family Euphorbiace. The plant is widely distributed throughout tropical Central America and South America and is widely recognized by ethnobotanists and local healers for its medicinal properties (McRae 1988), including for the treatment of diarrhea. Crofelemer is believed to exert its anti-diarrhea effect through luminal blockade of CFTR (cystic fibrosis transmembrane conductance regulator) chloride (Cl⁻) channel. Crofelemer has demonstrated in vitro activity against cholera toxin, forskolin, *E. coli* LT and STa toxin-mediated Cl⁻ secretion, and to normalize electrolyte and fluid accumulation in CF-treated mice (Gabriel 1999, Fischer 2004, Adam 2005) via its effects on the CFTR channel. Crofelemer also significantly improved the secretory diarrhea in humans due to enterotoxigenic *E. coli* (DiCesare 2002), which is also thought to evoke secretory diarrhea through activation of CFTR (Kunzelmann 2002). Blockade of the CFTR channel could be anticipated to have negative consequences in man, even mimicking cystic fibrosis. However, crofelemer has virtually no systemic bioavailability in humans. When studied, the results indicated that there was little or no absorption of crofelemer from the GI tract, and that crofelemer was well tolerated by normal male subjects. Thus, the site of action of crofelemer is topical in the gastrointestinal tract.

Crofelemer (CAS 148465-45-6) is an oligomeric proanthocyanidin of varying chain lengths derived from the Dragon's Blood *Croton lechleri* of the family Euphorbiaceae. Crofelemer has an average molecular weight of between approximately 1500 daltons and approximately 2900 daltons. The monomers comprising crofelemer comprise catechin, epicatechin, gallo catechin, and epigallo catechin. The chain length of crofelemer ranges from about 3 to about 30 units with an average chain length of about 8 units. The structure of crofelemer is shown below.



Wherein the average $n=6$.

Another method for isolating crofelemer can be found in U.S. Patent Publication No. 2005/0019389, the contents of which are expressly incorporated herein.

In other embodiments, a raw latex obtained from a *Croton* species or a *Calophyllum* species or an extract obtained from a *Croton* species or a *Calophyllum* species are useful in the methods presented herein. Exemplary extracts are described in Persinos et al., 1979, J. Pharma. Sci. 68:124 and Sethi, 1977, Canadian J. Pharm. Sci. 12:7.

"Ameliorate," "amelioration," "improvement" or the like refers to, for example, a detectable improvement or a detectable change consistent with improvement that occurs in a subject or in at least a minority of subjects, e.g., in at least about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 100% or in a range between about any two of these values. Such improvement or change may be observed in treated subjects as compared to subjects not treated with crofelemer, where the untreated subjects have, or are subject to developing, the same or similar disease, condition, symptom or the like. Amelioration of a disease, condition, symptom or assay parameter may be determined subjectively or objectively, e.g., self assessment by a subject(s), by a clinician's assessment or by conducting an appropriate assay or measurement. Amelioration may be transient, prolonged or permanent or it may be variable at relevant times during or after crofelemer is administered to a subject or is used in an assay or other method described herein or a cited reference, e.g., within timeframes described infra, or about 1 hour after the administration or use of crofelemer to about 7 days, 2 weeks, 28 days, or 1, 3, 6, 9 months or more after a subject(s) has received such treatment.

The "modulation" of, e.g., a symptom, level or biological activity of a molecule, or the like, refers, for example, that the symptom or activity, or the like is detectably increased or decreased. Such increase or decrease may be observed in treated subjects as compared to subjects not treated with crofelemer, where the untreated subjects have, or are subject to developing, the same or similar disease, condition, symptom or the like. Such increases or decreases may be at least

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about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 100%, 150%, 200%, 250%, 300%, 400%, 500%, 1000% or more or within any range between any two of these values. Modulation may be determined subjectively or objectively. Modulation may be transient, prolonged or permanent or it may be variable at relevant times during or after crofelemer is administered to a subject or is used in an assay or other method described herein or a cited reference, e.g., within times described infra, or about 1 hour of the administration or use of crofelemer to about 2 weeks, 28 days, 3, 6, 9 months or more after a subject(s) has received crofelemer.

The language "a prophylactically effective amount" of a compound refers to an amount of crofelemer which is effective, upon single or multiple dose administration to the subject, in preventing or treating diarrhea, e.g., HIV-associated diarrhea.

As used herein, "subject" includes organisms which are capable of suffering from HIV associated diarrhea or HAART associated diarrhea or who could otherwise benefit from the administration of crofelemer as described herein, such as humans and non-human animals. The term "non-human animals" includes all vertebrates, e.g., mammals such as non-human primates, other mammals, e.g., rodents, and sheep, dog, and cow, at risk for HIV associated diarrhea or HAART associated diarrhea. A subject at risk for HIV associated diarrhea or HAART associated diarrhea is meant to include a subject at risk of developing or contracting an HIV infection.

The language "a prophylactically effective amount" of a compound refers to an amount of crofelemer which is effective, upon single or multiple dose administration to the subject, in preventing or treating the diarrhea.

The term "administration" or "administering" includes routes of introducing crofelemer to a subject to perform their intended function. Examples of routes of administration that may be used include injection, oral, inhalation, vaginal, rectal and transdermal. The pharmaceutical preparations may be given by forms suitable for each administration route. For example, these preparations are administered in tablet or capsule form, by injection, inhalation, ointment, or suppository. Administration may also be by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral administration is preferred. Depending on the route of administration, crofelemer can be coated with or disposed in a selected material to protect it from natural conditions that may detrimentally affect its ability to perform its intended function. Crofelemer can be administered alone, or in conjunction with either another agent or agents as described above or with a pharmaceutically-acceptable carrier, or both. Exemplary enteric coated forms of crofelemer are described in, for example, U.S. Pat. No. 7,556,831.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration may vary depending upon the age, weight and mammalian species treated, the particular compounds employed, and/or the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods and in consultation with the data presented herein. In regard to crofelemer, it may be advantageous to administer 125 mg crofelemer two times per day to treat watery diarrhea if fewer watery stools

are desired over a week. It is also advantageous to treat with 500 mg two times per day if an improvement in stool consistency is desired.

The term "obtaining" as in "obtaining crofelemer" is intended to include purchasing, synthesizing, isolating, extracting or otherwise acquiring crofelemer.

The language "a prophylactically effective amount" of a compound refers to an amount of crofelemer which is effective, upon single or multiple dose administration to the subject, in preventing or treating an indication. In one embodiment, the indication is HIV-associated diarrhea.

Methods of Treatment

Provided herein are methods of treating, preventing, or alleviating diarrhea or the symptoms caused by HIV infection or HAART therapy for HIV infection comprising administering to a subject in need thereof an effective amount of crofelemer. Exemplary diarrhea that can be treated or prevented using the methods presented herein include HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea.

In one embodiment, treating HIV-associated diarrhea includes an improvement of the following symptoms of HIV associated diarrhea or HAART associated diarrhea, including, for example, a decrease in the number of bowel movements per day (frequency), a decrease in the number of watery bowel movements per day, a decrease in symptom frequency (urgency, fecal incontinence), a decrease in symptom severity (abdominal pain or discomfort), a decrease in daily stool consistency score (watery to formed), or a decrease in stool consistency leading to formed stools from watery stools.

In one specific embodiment, treatment is defined as two or less watery bowel movements per week. More specifically, treatment is defined as two or less watery bowel movements per week during at least two of the four weeks of treatment with crofelemer.

In other specific embodiments, treatment can also include, for example, one or more of a decrease in the number of bowel movements per day, a decrease in the number of watery bowel movements per day, an improvement in the daily abdominal score for pain or discomfort, an improvement in the score for daily stool consistency, a decrease in the number of days per week that subjects experienced urgency, a decrease in the number of days per week that subjects experienced fecal incontinence, or a decrease in the unscheduled visit for a significant worsening of diarrhea.

In one aspect, provided herein are methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject. Treatment of the diarrhea can include, for example, one or more of a decrease in the number of bowel movements per day, a decrease in the number of watery bowel movements per day, an improvement in the daily abdominal score for pain or discomfort, an improvement in the score for daily stool consistency, a decrease in stool consistency, a decrease in the number of days per week that subjects experienced urgency, a decrease in the number of days per week that subjects experienced fecal incontinence, or a decrease in the unscheduled visit for a significant worsening of diarrhea.

In one aspect, provided herein are methods of treating stool consistency in an HIV positive subject, wherein a subject is considered treated if there is an improvement in the score for daily stool consistency and/or a decrease in stool consistency score as measured throughout the day or days or weeks. This decrease may be measured from a baseline. The baseline may be determined in the days to week prior to treatment with crofelemer. Treatment comprises administering about 250 mg to about 1000 mg per day; administering about 250 mg per

day; administering 1000 mg per day; administering about 125 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, provided herein are methods of improving stool consistency in an HIV positive subject wherein a subject is considered treated if there is an improvement in stool consistency and/or a decrease in stool consistency throughout the day or days or weeks. This increase may be measured from a baseline. The baseline may be determined in the days to week prior to treatment with crofelemer. Treatment comprises administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering 1000 mg per day; administering about 125 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, provided herein are methods of alleviating watery diarrhea in an HIV positive subject, wherein a subject is considered treated if the subject experiences a decrease in the number of watery bowel movements per day and/or over days, a week or weeks of administration of crofelemer. This decrease may be measured from a baseline. The baseline may be determined in the days to week prior to treatment with crofelemer. Treatment comprises administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering 1000 mg per day; administering about 125 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

In one aspect, presented herein are methods of decreasing the number of bowel movements per day, wherein a subject is considered treated if there is a decrease in the number of bowel movements per day as measured from a baseline. The baseline may be determined in the days to week prior to treatment with crofelemer. Treatment comprises administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering 1000 mg per day; administering about 125 mg two times per day; or administering about 500 mg two times per day of crofelemer to a subject in need thereof.

Crofelemer may be administered, for example, once a day, twice a day, three times a day, or four times or more often as necessary per day. Crofelemer may be administered in doses, for example of from about between 25 mg BID to about 3000 mg TID, preferably crofelemer is administered from between about 125 mg to about 1000 mg per day. In another embodiment, crofelemer is administered between 125 mg BID to about 500 mg BID depending of symptoms. In another embodiment, crofelemer is administered as 125 mg BID. In another embodiment, crofelemer is administered as 500 mg BID. Crofelemer may be administered orally, for example, in tablet form, powdered form, liquid form or in capsules.

In exemplary embodiments, the subject is administered 250, 500, or 1000 mg/day of crofelemer.

In specific embodiments, the subject is administered 125, 250 or 500 mg p.o. b.i.d (orally twice per day). Other appropriate dosages for methods may be determined by health care professionals or by the subject. The amount of crofelemer administered daily may be increased or decreased based on the weight, age, health, sex or medical condition of the subject. One of skill in the art would be able to determine the proper dose for a subject based on this disclosure and the data presented in the Examples, which follow.

In other embodiments, the subject is treated with crofelemer for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 or more weeks or 26 or more weeks. In one embodiment, the subject is administered crofelemer for the duration of the disease. Length of treatment may vary depending on the type and length of disease and the proper length of treatment may be

easily determined by one of skill in the art having the benefit of this disclosure. Treatment may be prior to infection, during infection, or after suspected infection and for a period of time suggested by a medical professional to reduce or eliminate diarrhea.

Subjects in need thereof include subjects having or that are susceptible to or who have HIV-associated diarrhea or HAART associated diarrhea.

As used herein, a therapeutically effective amount means an amount effective, when administered to a human or non-human subject, to provide a therapeutic benefit such as an amelioration of symptoms, e.g., an amount effective to decrease the symptoms HIV-associated diarrhea.

According to certain embodiments, crofelemer may be administered in combination with other compounds, including for example, anti-diarrheal agents or anti-HIV agents, including, for example, anti-retroviral agents.

According to one aspect, provided herein are methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject that has previously used protease inhibitors, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a male subject in need thereof. As used herein, "previously used" includes, for example, subjects who have used protease inhibitors (PIs) prior to crofelemer therapy or overlapping with crofelemer therapy, but the PI use began prior to the first dose of crofelemer therapy.

Pharmaceutical Preparations

Also provided herein are pharmaceutical compositions, comprising an effective amount of a crofelemer described herein and a pharmaceutically acceptable carrier. In a further embodiment, the effective amount is effective to treat HIV-associated diarrhea and/or HAART associated diarrhea.

Examples of the preparation and use of crofelemer have been described in U.S. Pat. No. 7,556,831, US Patent Publication 20070254050 and US Patent Publication 20080031984, all of which are incorporated herein by reference in their entirety.

One embodiment includes pharmaceutical compositions comprising crofelemer and a pharmaceutically acceptable carrier.

The pharmaceutical compositions described herein may further comprise excipients, for example, one or more of a diluting agent, binding agent, lubricating agent, disintegrating agent, coloring agent, flavoring agent or sweetening agent. Compositions may be formulated for selected coated and uncoated tablets, hard and soft gelatin capsules, sugar-coated pills, lozenges, wafer sheets, pellets and powders in sealed packet. For example, compositions may be formulated for topical use, for example, ointments, pomades, creams, gels and lotions.

In certain embodiments, these pharmaceutical compositions are suitable for topical or oral administration to a subject. In other embodiments, as described in detail below, the pharmaceutical compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for

example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

The phrase "pharmaceutically acceptable" refers to crofelemer as described herein, compositions containing crofelemer, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" includes pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Compositions containing crofelemer include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from

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about 0.01% to about 99% of active ingredient, for example, from about 5% to about 70%, or from about 10% to about 30%.

Liquid dosage forms for oral or rectal administration of crofelemer may include, for example, pharmaceutically-acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Suspensions, in addition to crofelemer may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof. Dosage forms for the topical or transdermal administration of crofelemer can include, for example, powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The ointments, pastes, creams and gels may contain, in addition to crofelemer, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof. Powders and sprays can contain, in addition to a crofelemer, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Examples of suitable aqueous and non-aqueous carriers which may be employed in the pharmaceutical compositions can include, for example, water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

In one embodiment, crofelemer is enteric coated so as to protect it from degradation by the acidic conditions of the stomach and/or from interactions with proteins, such as pepsin, present in the stomach, e.g., an enteric protected formulation. In a specific embodiment, crofelemer is in tablet form. In yet another embodiment, the tablet is enteric coated, e.g., Eudragit®. In one embodiment, crofelemer is formulated as an enteric coated bead or granule in an enteric coated capsule shell. In another embodiment, crofelemer is formulated in a delayed release composition.

In certain embodiments, the composition is formulated with a compound or compounds which neutralize stomach acid. Alternatively, the pharmaceutical composition containing the composition is administered either concurrent with or subsequent to or after administration of a pharmaceutical composition which neutralize stomach acid. Compounds, such as antacids, which are useful for neutralizing stomach acid include, but are not limited to, aluminum carbonate, aluminum hydroxide, bismuth subnitrate, bismuth subsalicylate, calcium carbonate, dihydroxyaluminum sodium carbonate, magaldrate, magnesium carbonate, magnesium hydroxide, magnesium oxide, and mixtures thereof. Compounds that

are able to reduce the secretion of stomach acid and/or are able to reduce the acidity of stomach fluid are well known in the art and include, but are not limited to, antacids (aluminum hydroxide, aluminum carbonate, aluminum glycinate, magnesium oxide, magnesium hydroxide, magnesium carbonate, calcium carbonate, sodium bicarbonate), stomach acid blockers and a combination of any of the foregoing. In general, any drug that has been approved for sale by the relevant government agency and is able to reduce the production of stomach acid and/or reduce the acidity of stomach fluid can be administered in combination with an inhibitor molecule, such as crofelemer, in accordance with the methods presented herein.

In a particular embodiment where crofelemer is not enteric coated, crofelemer is formulated with one or more compounds that are able to reduce the secretion of stomach acid and/or able to reduce the acidity of stomach fluid. In an exemplary embodiment, crofelemer is formulated in a controlled release (delayed release) composition, such as Merck GEM, Alza OROS, wax matrix (release is primarily delayed until after the formulation passes out of the stomach and into the intestine).

Also provided herein are pharmaceutical formulations of crofelemer comprising the composition along with a pharmaceutically acceptable vehicle, at a dose which is therapeutically effective at treating HIV associated diarrhea or HAART associated diarrhea. In one embodiment, a directly compressible crofelemer (e.g., that can be directly compressed, without excipients, into a tablet of pharmaceutically acceptable hardness and friability) compressed into a tablet, optionally with a lubricant, such as but not limited to magnesium stearate, is enteric coated. These formulations can be prepared by methods known in the art, see, e.g. methods described in Remington's Pharmaceutical Sciences, 18th Ed., ed. Alfonso R. Gennaro, Mack Publishing Co., Easton, Pa., 1990.

In a specific embodiment, the proanthocyanidin polymer composition comprises crofelemer (CAS 148465-45-6).

In a more another embodiment, a composition is enteric coated. Enteric coatings are those coatings that remain intact in the stomach, but will dissolve and release the contents of the dosage form once it reaches the small intestine. A large number of enteric coatings are prepared with ingredients that have acidic groups such that, at the very low pH present in the stomach, i.e. pH 1.5 to 2.5, the acidic groups are not ionized and the coating remains in an undissociated, insoluble form. At higher pH levels, such as in the environment of the intestine, the enteric coating is converted to an ionized form, which can be dissolved to release the inhibitor molecule. Other enteric coatings remain intact until they are degraded by enzymes in the small intestine, and others break apart after a defined exposure to moisture, such that the coatings remain intact until after passage into the small intestines.

Polymers which are useful for the preparation of enteric coatings include, but are not limited to, shellac, starch and amylose acetate phthalates, styrene-maleic acid copolymers, cellulose acetate succinate, cellulose acetate phthalate (CAP), polyvinylacetate phthalate (PVAP), hydroxypropylmethylcellulose phthalate (grades HP-50 and HP-55), ethylcellulose, fats, butyl stearate, and methacrylic acid-methacrylic acid ester copolymers with acid ionizable groups. In one embodiment, the pharmaceutical composition contains a polymeric proanthocyanidin composition and the enteric coating polymer Eudragit®. L 30D, an anionic copolymer of methacrylic acid and methyl acrylate with a mean molecular weight of 250,000 Daltons. In another embodiment, the enteric coating polymer is Eudragit®. L 30D-55. Application of the enteric coating to the crofelemer composition can be accomplished by any method known in the art for applying

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enteric coatings. For example, but not by way of limitation, the enteric polymers can be applied using organic solvent based solutions containing from 5 to 10% w/w polymer for spray applications and up to 30% w/w polymer for pan coatings. Solvents that are commonly in use include, but are not limited to, acetone, acetone/ethyl acetate mixtures, methyl-
cne chloride/methanol mixtures, and tertiary mixtures containing these solvents. Some enteric polymers, such as meth-
acrylic acid-methacrylic acid ester copolymers can be applied using water as a dispersant. The volatility of the solvent
system must be tailored to prevent sticking due to tackiness and to prevent high porosity of the coating due to premature
spray drying or precipitation of the polymer as the solvent evaporates.

In another embodiment, the pharmaceutical composition comprising crofelemer is formulated as enteric coated gran-
ules or powder (microspheres with a diameter of 300-5001) provided in either hard shell gelatin capsules or suspended in
an oral solution for pediatric administration. The enteric coated powder or granules may also be mixed with food,
particularly for pediatric administration.

The granules and powder can be prepared using any method known in the art, such as but not limited to, crystal-
lization, spray-drying or any method of comminution, for example, using a high speed mixer/granulator. Exemplary
formulations may be found, for example, in the following US patents and applications U.S. Pat. No. 7,341,744; U.S. Ser.
No. 11/510,152; and U.S. Ser. No. 12/175,131.

Regardless of the route of administration selected, crofele-
mer is formulated into pharmaceutically-acceptable dosage forms by methods known to those of skill in the art.

In combination therapy treatment, both the compounds and the other drug agent(s) are administered to mammals (e.g.,
humans, male or female) by methods known in the art. The agents may be administered in a single dosage form or in
separate dosage forms. Effective amounts of the other thera-
peutic agents are well known to those skilled in the art. How-
ever, it is well within the skilled artisan's purview to deter-
mine the other therapeutic agent's optimal effective-amount
range. In one embodiment in which another therapeutic agent
is administered to an animal, the effective amount of the
compound is less than its effective amount in case the other
therapeutic agent is not administered. In another embodi-
ment, the effective amount of the agent is less than its effec-
tive amount in case the compound is not administered. In this
way, undesired side effects associated with high doses of
either agent may be minimized. Other potential advantages
(including without limitation improved dosing regimens and/
or reduced drug cost) will be apparent to those skilled in the
art.

In various embodiments, the therapies (e.g., prophylactic
or therapeutic agents) are administered less than 5 minutes
apart, less than 30 minutes apart, 1 hour apart, at about 1 hour
apart, at about 1 to about 2 hours apart, at about 2 hours to
about 3 hours apart, at about 3 hours to about 4 hours apart, at
about 4 hours to about 5 hours apart, at about 5 hours to about
6 hours apart, at about 6 hours to about 7 hours apart, at about
7 hours to about 8 hours apart, at about 8 hours to about 9
hours apart, at about 9 hours to about 10 hours apart, at about
10 hours to about 11 hours apart, at about 11 hours to about 12
hours apart, at about 12 hours to 18 hours apart, 18 hours to 24
hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours
apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart,
60 hours to 72 hours apart, 72 hours to 84 hours apart, 84
hours to 96 hours apart, or 96 hours to 120 hours part. In one
or more embodiments, two or more therapies are adminis-
tered within the same patient's visit.

Kits

Kits are also provided herein, for example, kits for treating
a diarrhea, e.g., HIV-associated diarrhea in a subject. The kits
may contain, for example, crofelemer or a pharmaceutical
composition comprising crofelemer and instructions for use.
The instructions for use may contain prescribing information,
dosage information, storage information, and the like.

Label instructions include, for example, instructions to
take the crofelemer for at least 3 days for the treatment of HIV
associated or HAART associated diarrhea. The instructions
could also read, for example, take from between 125 mg BID
to 500 mg BID of crofelemer until resolution of symptoms or
for 24 weeks for treatment or HIV-associated diarrhea. The
instructions could also read, for example, take 125 mg BID of
crofelemer until resolution of symptoms or for 24 weeks for
treatment or HIV-associated diarrhea. The instructions could
also read, for example, take 500 mg BID of crofelemer until
resolution of symptoms or for 24 weeks for treatment or
HIV-associated diarrhea.

Label instructions may further or alternately include, for
example, instructions to take the crofelemer from between
125 mg BID to 500 mg BID of crofelemer until resolution of
symptoms or for 26 weeks for treatment or HIV-associated
diarrhea. The instructions could also read, for example, take
125 mg BID of crofelemer until resolution of symptoms or for
26 weeks for treatment or HIV-associated diarrhea. The
instructions could also read, for example, take 250 mg BID of
crofelemer until resolution of symptoms or for 26 weeks for
treatment or HIV-associated diarrhea. The instructions could
also read, for example, take 500 mg BID of crofelemer until
resolution of symptoms or for 26 weeks for treatment or
HIV-associated diarrhea.

EXAMPLES

It should be appreciated that the invention should not be
construed to be limited to the example, which is now
described; rather, the invention should be construed to
include any and all applications provided herein and all
equivalent variations within the skill of the ordinary artisan.

Example 1

Pulmonary Effects of Orally Administered
Crofelemer in Rats

Three treatment groups of eight male rats were adminis-
tered Crofelemer at respective dose levels of 60, 200, and 600
mg/kg. An additional group of eight male rats served as con-
trol animals and were administered the vehicle, purified
water. Crofelemer and vehicle were administered at a dose
volume of 10 mL/kg. One additional group of eight male rats
received the positive control article, baclofen, at a dose level
of 100 mg/kg and a dose volume of 15 mL/kg. Crofelemer,
positive control article, and vehicle were administered to all
groups via oral gavage.

Observations for mortality, morbidity, injury, and the avail-
ability of food and water were conducted at least twice daily
for all animals. Clinical observations were conducted prior to
dosing, approximately 1 hour postdose, and following
completion of the pulmonary monitoring periods (approx-
imately 4 hours postdose). Body weights were measured and
recorded prior to dosing (Day 1). Pulmonary function (respi-
ratory rate, tidal volume, and minute volume) was monitored
for approximately 1 hour prior to dosing to establish baseline
and for approximately 4 hours postdose. Following the pul-

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monary monitoring periods, all animals were euthanized and the carcasses were discarded without further evaluation.

Crofelemer, administered orally to male rats at doses of 60, 200, and 600 mg/kg did not produce any effects on mortality or any of the quantitative respiratory endpoints over the course of the study. With respect to the basic respiratory endpoints evaluated in this study, oral administration of Crofelemer produced no adverse effects in rats at doses up to and including 60 mg/kg.

Example 2

13-Week Oral Toxicity Study of Crofelemer Administered to Mice

Three treatment groups of 15 male and 15 female mice were administered Crofelemer at respective dose levels of 40, 400, and 1200 mg/kg/day. One additional group of 15 animals/sex served as the control and received the vehicle, purified water. The vehicle or Crofelemer was administered to all groups at a dose volume of 10 mL/kg. Additionally, four groups of eight or 39 animals/sex/group served as toxicokinetic (TK) animals and received the control or Crofelemer in the same manner as the main study groups at respective dose levels of 0, 40, 400, or 1200 mg/kg/day. Due to mortalities, the main study and TK animals at 1200 mg/kg/day were administered Crofelemer for up to 55 or 56 days, respectively.

Observations for morbidity, mortality, injury, and the availability of food and water were conducted twice daily for all animals. Detailed clinical observations for clinical signs were conducted weekly on all main study animals. Body weights were measured and recorded weekly on all animals. Food consumption was measured and recorded weekly on all main study animals. Ophthalmoscopic examinations were conducted pretest and prior to necropsy for all main study animals. Blood samples for clinical pathology evaluations were collected from main study animals in extremis and at termination. Blood samples for determination of the plasma concentrations of Crofelemer were collected from designated TK animals at designated time points on Days 1, 56, and 91. After blood collection, the TK animals were euthanized and the carcasses were discarded, with the exception of designated animals at 1200 mg/kg/day. The toxicokinetic parameters were determined for Crofelemer from concentration time data in the test species. At study termination, necropsy examinations were performed and organ weights were recorded for all main study animals and designated TK animals at 1200 mg/kg/day. Tissues were microscopically examined for main study animals at 0, 400, and 1200 mg/kg/day. Beginning on Day 7, a limited gross necropsy examination was performed on any TK animal euthanized in extremis or found dead in an effort to determine the cause of death. Tissues were collected and preserved for possible future examination from main study animals at 40 mg/kg/day and designated TK animals at 1200 mg/kg/day.

Twice-daily oral gavage administration of Crofelemer at 0, 40, or 400 mg/kg/day for 13 weeks or 1200 mg/kg/day for 8 weeks in mice was only tolerated in females at 40 mg/kg/day. Crofelemer-related mortalities were evident in a single male at 40 mg/kg/day and in both sexes at 400 and 1200 mg/kg/day. Crofelemer-related body weight effects were evident at ≥ 400 mg/kg/day in both sexes, and food consumption effects were evident at 40 mg/kg/day in females and ≥ 400 mg/kg/day in both sexes. Clinical pathology, organ weight, and macroscopic effects were observed in both sexes at 1200 mg/kg/day. Due to mortality at 40 mg/kg/day, there was no No-Observed-

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Adverse-Effect-Level (NOAEL) in males; however, the NOAEL was determined to be 40 mg/kg/day in females.

Example 3

Neurobehavioral Evaluation of Orally Administered Crofelemer in Rats

Three treatment groups of six male rats were administered Crofelemer at respective dose levels of 60, 200, and 600 mg/kg. One additional group of six male rats served as the control and received the vehicle, purified water. Another additional group of six male rats received the positive control article, chlorpromazine hydrochloride, at a dose level of 20 mg/kg. Crofelemer, positive control article, or vehicle was administered to all groups once via oral gavage at a dose volume of 10 mL/kg.

Observations for morbidity, mortality, injury, and the availability of food and water were conducted at least twice daily for all animals. Clinical observations were conducted following each functional observational battery (FOB) examination. FOB evaluations were conducted predose and at approximately 1 and 24 hours postdose. Body weights were measured and recorded prior to dosing on Day 1. At study termination, all animals were euthanized and the carcasses were discarded without further evaluation.

Crofelemer administered orally to male rats at doses of 60, 200, and 600 mg/kg did not produce any effects on mortality, clinical observations, body weight, or any of the neurobehavioral measures tested. Therefore, with respect to the basic neurobehavioral endpoints evaluated in this study, oral administration of Crofelemer produced no effects in rats at doses up to and including 600 mg/kg.

Example 4

Effects of Orally Administered Crofelemer on Gut Motility Function in the Rat

Three treatment groups of eight male rats were administered Crofelemer at respective dose levels of 60, 200, and 600 mg/kg. One additional group of eight male rats received the positive control article, morphine, at a dose level of 20 mg/kg. An additional group of eight male rats served as the control and received the vehicle, purified water. The vehicle, positive control article, or Crofelemer was administered to all groups via oral gavage once on Day 1 of the study at a dose volume of 10 mL/kg. Approximately 1 hour after administration, the test meal, 5% charcoal suspension in 10% *Acacia* in deionized water, was administered to all animals via oral gavage at a dose volume of 10 mL/kg.

Observations for morbidity, mortality, injury, and the availability of food (except during fasting periods) and water were conducted at least twice daily for all animals. Clinical observations were conducted prior to dosing, and prior to termination. Body weights were measured and recorded prior to dosing on Day 1. Approximately 30 minutes following test meal administration all animals were euthanized, the small intestine was surgically removed, and the total intestine length and the distance the charcoal traveled were both measured. The carcasses were discarded without further evaluation.

Crofelemer, administered orally to male rats at doses of 60, 200, or 600 mg/kg did not produce mortality or any clinical observations. Crofelemer-related, dose-dependent decreases in gastrointestinal propulsion were noted in all Crofelemer-treated groups; however, statistically significant decreases

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were only noted following the 200 and 600 mg/kg doses. Due to low recovery values, the dose levels actually administered to the animals in the 60 and 200 mg/kg groups were 51 and 169 mg/kg, respectively.

Example 5

Cardiovascular Effects of Orally Administered Crofelemer in the Beagle Dog

The same four male beagle dogs were administered the control article, placebo tablets in gelatin number 12 Torpac lock ring gelatin capsules (0 mg/kg), and Crofelemer at dose levels of approximately 60, 200, and 600 mg/kg according to a modified Latin square design, with one animal/sex/treatment dosing each week followed by at least a 7 day washout period between administrations, until each animal received all treatments. The control article and Crofelemer were administered to all animals orally via gelatin capsule.

The animals were previously surgically instrumented with radio transmitters for measurement of body temperature, blood pressure, heart rate, and the electrocardiogram (ECG). Body temperature, systolic, diastolic, and mean arterial blood pressures, heart rate, and ECG parameters (QRS duration and the RR, PR, and QT intervals) were monitored continuously from at least 2 hours prior to dosing until at least 20 hours postdose. Nine days prior to the first administration, untreated animals were continuously monitored for cardiovascular endpoints for at least 22 hours. These data were used in the calculation of the corrected QT interval throughout the study.

Observations for morbidity, mortality, injury, and the availability of food and water were conducted at least twice daily for all animals. Clinical observations were conducted prior to dosing and following completion of the cardiovascular monitoring period. Body weights were measured and recorded on the day prior to each administration. At study termination, the animals were transferred to the stock colony.

Crofelemer administered orally to male dogs at doses of 60, 200, and 600 mg/kg did not produce mortality nor any effects on the ECG over the course of the study. Following doses of 200 and 600 mg/kg the test article produced clinical observations of red, black, or brown feces; soft, watery, and/or mucoid feces; and/or black or brown material below the cage (fecal material). Therefore, with respect to all the physiological parameters evaluated as a part of this cardiovascular study, a no-observable-adverse-effect-level (NOAEL) of 600 mg/kg has been established.

Example 6

Effects of Crofelemer on Herg K⁺ Currents in HEK-293 Cells

The hERG channel was stably expressed in a subclone (HEK-293/hERG) of the HEK-293 cell line. The effect of Crofelemer was measured on the maximum amplitude of the tail current. This parameter was determined from current traces obtained from voltage-clamped HEK-293/hERG cells, using patch-clamp techniques in the whole cell configuration.

Crofelemer was tested at 5 concentrations for the first set of experiments: 0.001 μ M, 0.01 μ M, 0.1 μ M, 1.0 μ M, and 10.0 μ M and 6 concentrations for the second set of experiments: 0.1 μ M, 0.3 μ M, 1 μ M, 3 μ M, 10 μ M, and 30 μ M. Positive control article was tested at 10 μ M. Negative control article was deionized water.

Crofelemer inhibited hERG tail current in a dose-dependent manner. The estimated IC₅₀ values were 1.79 μ M for the

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first set of experiments and 1.75 μ M for the second set of experiments. Cisapride (positive control, 10 μ M) inhibited hERG tail current by an average of 99.67% and 100.47% for the first and second sets of experiments respectively, which is consistent with its known pharmacological action.

Example 7

Effects of Food on the PK of Crofelemer 500 mg

A total of 28 subjects were to be enrolled into this study. Subjects were randomized on Day 1 at a 1:1 ratio to Group 1 (fasted then fed) or Group 2 (fed then fasted). Randomization was stratified by sex. Each subject received a single dose of crofelemer 500 mg (administered orally as 2x250 mg tablets) with a high fat meal (crofelemer fed) and after fasting (crofelemer fasted). The fasted/fed study periods were separated by 7 days. The sequence of fasted/fed or fed/fasted dosing on Days 1 and 8 were determined by randomization on Day 1.

Blood samples for PK analyses of crofelemer were collected pre-dose and up to 48 hours post-dose following both fasted and fed single-dose treatment administrations.

During the food effect study period, subjects fasted overnight (no food for approximately 9.5 hours) prior to administration of a high fat breakfast (crofelemer fed) or fasted overnight for 10 hours prior to administration of the single dose of study drug (crofelemer fasted).

Assessments of the treatment regimen on the relative bioavailability of crofelemer were based on comparisons of plasma concentrations of crofelemer across each study phase. Whole blood samples were drawn at the following times (actual blood collection times were recorded in the source/eCRF): Days 1 and 8: pre-dose (approximately 1 hour before dosing), and at 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 24, 30, 36, 42, and 48 hours post-dose.

A pharmacokinetic/pharmacodynamic (PK/PD) analysis was intended to be performed using all subjects who had paired ECG and plasma concentrations for crofelemer. The PK/PD pharmacokinetic-pharmacodynamic analysis was not done due to insufficient pharmacokinetic PK data.

This analysis was also designed to assess the relationship between the concentration of crofelemer and QTcF. However, only 3 samples were found to have concentrations above the LLOQ of 50 ng/mL so the relationship of crofelemer concentration to QTcF could not be assessed.

The mean baseline corrected change in heart rate showed a decrease in heart rate of -1.1 bpm and -1.0 bpm for crofelemer fasting and fed, respectively, at 4 hours. The mean baseline corrected change in heart rate showed an increase in heart rate of 3.5 bpm and 1.6 bpm for crofelemer fasting and fed, respectively, at 12 hours. The heart rate changes were of no clinical significance. There were no tachycardic or bradycardic outliers on crofelemer fasting or fed.

Example 8

Efficacy and Safety of Crofelemer for the Treatment of HIV Associated Diarrhea

This study was conducted to determine if treatment with Crofelemer 125 mg, 250 mg and 500 mg orally (p.o.) twice daily improves the frequency, consistency, and urgency of bowel movements in subjects with HIV-associated diarrhea compared to placebo. Male or female subjects aged $\geq$ 18 years, with HIV-1 infection confirmed by standard serological tests and/or viral load and history of diarrhea of at least 1 month duration.

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The study was a randomized, double-blind, parallel-group, placebo-controlled, multicenter study. The study was done in two stages. Both stages consisted of a 10+4 day, single-blind, placebo screening phase; followed by randomization and a 31-day, double-blind, placebo-controlled treatment phase; and concluded with a 20-week placebo-free extension phase. In Stage I (dose selection stage), the double-blind phase had four arms: three doses of crofelemer (125 mg b.i.d., 250 mg b.i.d., and 500 mg b.i.d.) and placebo b.i.d. The chance of receiving crofelemer or placebo was 3:1 (1:1:1:1 ratio). Subjects on crofelemer rolling over into the extension phase remained on their same dose, or if on placebo was re-randomized to one of the three aforementioned doses. After Stage I is completed an interim analysis was conducted and the dose of crofelemer that appears to work better, be safer, and/or better tolerated than the others, was selected to use exclusively in Stage II. Stage II will have only two arms in the double blind, placebo-controlled treatment phase: the selected dose of crofelemer (crofelemer 125 mg) and placebo. The chances of receiving crofelemer or placebo will be 1:1. All subjects rolling over into the 20-week placebo-free extension phase in Stage II were assigned the previously selected dose of crofelemer. During both Stage I and Stage II, subjects will first enter a single-blind placebo screening phase lasting 10+4 days during which time bowel movement frequency, consistency, and urgency was measured. Antiretroviral therapy and therapy for associated conditions (including prophylactic antibiotics for *Pneumocystis carinii* (PCP) or infection must have remained at a constant level from four weeks prior to screening through the placebo-controlled treatment phase. Any changes in antiretroviral therapy during any point in the study must be reported to the site and documented on the subject's case report form.

The key efficacy analyses were based on the data from the 4-week efficacy assessment period of the placebo-controlled treatment phase. Analysis of the primary endpoint was based on the Intent to Treat (ITT) population and will compare the proportion of responders in the placebo group to the proportion of responders in the crofelemer 125 mg group. The primary efficacy endpoint was clinical response, defined as two or less watery bowel movements per week, during at least two of the four weeks of the 4-week efficacy assessment period in the ITT population. The secondary efficacy variables during the 4-week efficacy assessment period in the ITT population were:

- The number of bowel movements per day
- The number of watery bowel movements per day;
- The score for daily abdominal pain or discomfort;
- The score for daily stool consistency;
- The number of days per week that subjects experienced urgency;
- The number of days per week that subjects experienced fecal incontinence; and
- Proportion of subjects undergoing an unscheduled visit for a significant worsening or clinically significant exacerbation of diarrhea during the 4-week efficacy assessment period.

This study consisted of a dose-selection stage, an interim analysis period, and a dose-assessment stage.

Stage I: Dose-Selection Stage

Subjects were randomized 1:1:1:1, at approximately 50 subjects per treatment group: crofelemer 125 mg p.o. b.i.d.; crofelemer 250 mg p.o. b.i.d.; crofelemer 500 mg p.o. b.i.d.; and placebo p.o. b.i.d.

Crofelemer 125 mg, 250 mg, and 500 mg, or matching placebo, was administered as a tablet combination orally twice daily with fluids at least one half hour before the morning and evening meals.

The double-blind, placebo-controlled treatment phase consisted of an initial 3-day run-in period (Days -3 to -1) followed by a 4-week efficacy assessment period (Days 1 to 28). The run-in period assured that the effects of study medication were established before the 4-week efficacy assessment period is commenced.

Subjects who completed the placebo-controlled treatment phase entered a 20-week placebo-free extension phase. Subjects in the Crofelemer 125 mg p.o. b.i.d., Crofelemer 250 mg p.o. b.i.d or Crofelemer 500 mg b.i.d. groups continued to receive these therapies throughout the placebo-free treatment phase; subjects who received placebo were re-randomized to receive either Crofelemer 125 mg p.o. b.i.d, Crofelemer 250 mg p.o. b.i.d. or 500 mg p.o. b.i.d (1:1:1). There was no risk of placebo during the 20-week extension phase, and subjects were permitted ad libitum (prn) use of ADM.

Stage I ended when about 50 subjects were randomized to each of the four treatment groups. Enrollment was stopped at approximately 50 subjects per treatment group until the interim analysis and decision for Stage II are completed. Interim Analysis

An interim analysis was performed when approximately 50 subjects (actual totals in the Tables, which follow) were randomized to each of four treatment groups and completed the placebo-controlled treatment period or terminate the study (not in either case to include the 14-day post-dosing telephone call for assessment of adverse events). Based upon an assessment of efficacy and safety, subjects were select to continue one of the crofelemer doses along with placebo into Stage II.

Stage II: Dose-Assessment Stage

Once the interim analysis was completed, enrollment resumed. Subjects were screened for eligibility for randomization based on the criteria in Stage I. Subjects who enter the double-blind, placebo-controlled treatment phase in Stage II were randomized to one of two treatment groups: crofelemer 125 mg p.o. b.i.d. or placebo p.o. b.i.d.

Crofelemer 125 mg, 250 mg, and 500 mg, or matching placebo, was administered as a tablet combination orally twice daily with fluids at least one half hour before the morning and evening meals.

An objective of the dose-assessment stage was to determine the proportion of HIV-positive subjects experiencing relief of diarrhea with crofelemer 125 mg p.o. b.i.d. compared to placebo p.o. b.i.d. during the placebo-controlled treatment phase. Other objectives were to evaluate the effects of crofelemer 125 mg p.o. b.i.d. vs. placebo on:

- i. Number of bowel movements per day (frequency)
- ii. Number of watery bowel movements per day
- iii. Symptom frequency (urgency, fecal incontinence)
- iv. Symptom severity (abdominal pain or discomfort); and
- v. Daily stool consistency score.

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The ratio of randomization to crofelemer 125 mg p.o. b.i.d or placebo p.o. b.i.d was 1:1. Subjects who completed the double-blind treatment phase participated in the 20-week, placebo-free extension phase and received crofelemer 125 mg p.o. b.i.d. Subjects who enrolled in Stage I, and who received either crofelemer 125 mg p.o. b.i.d., 250 mg p.o. b.i.d. or 500 mg p.o. b.i.d, remained on their previously assigned dose. However, subjects were re-assigned to crofelemer 125 mg p.o. b.i.d. if, in the opinion of the investigator, the response to or tolerance to their current dose was inadequate. Treatment remained blinded during this treatment period, including the possibility that subjects were switched to the same dose they had initially been taking.

All study procedures performed in Stage I were otherwise be identical to Stage II.

Subjects randomized during Stages I and II were combined and included in sample size calculations and analysis of efficacy and safety.

The following criteria were used for collecting data to evaluate the efficacy of crofelemer on HIV-associated diarrhea.

Study Diary (IVRS) Definitions

Diarrhea, includes, frequent loose or watery bowel movements

Bowel movement is defined as a trip to the bathroom with evacuation of stool; number of bowel movements means number of trips to the bathroom with evacuation of stool.

Watery bowel movement is defined as stool that can be poured;

Loose bowel movement is defined as soft blobs with no shape or form;

Formed bowel movement is defined as a stool like a soft sausage;

Hard bowel movement is defined as a stool like a hard or lumpy sausage; and

Very hard bowel movement is defined as hard lumps or nuts that are hard to pass.

Urgency is defined as having to rush to the bathroom for a bowel movement

Fecal incontinence is defined as leaking or passing stool at unwanted times (two teaspoons or more of stool).

Abdominal pain or discomfort is defined as pain, cramping, or bloating that is uncomfortable and/or interrupts normal activities.

Stool Samples

Each sample collected was analyzed as follows:

Visit 0

Clostridium difficile toxin;

Enteric pathogens, O&P examination;

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Giardia-specific antigen by EIA;

Modified acid-fast stain for *Cryptosporidium*, *Cyclospora*, and *Isospora*;

Lactoferrin (qualitative); and

Occult blood.

Visit 3

Clostridium difficile toxin;

Enteric pathogens, O&P examination;

Giardia-specific antigen by EIA; and

Modified acid-fast stain for *Cryptosporidium*, *Cyclospora*, and *Isospora*.

Visits 4, 5, 6, 7 and 8

Clostridium difficile toxin;

Enteric pathogens, O&P examination

Giardia-specific antigen by EIA

Modified acid-fast stain for *Cryptosporidium*, *Cyclospora*, and *Isospora*

Analysis of the Primary Efficacy Variable

The primary efficacy endpoint is clinical response; subjects are classified responders if they reported two or less watery bowel movements per week, during at least two of the four weeks of the efficacy assessment period of the placebo-controlled treatment phase.

Analysis of Secondary Efficacy Variables

For every subject, a mean baseline, a mean for Weeks 1 to 4, and change from baseline will be calculated for the following variables:

Number of bowel movements per day;

Number of watery bowel movements per day;

Daily abdominal pain or discomfort score;

Daily stool consistency score;

The number of days per week that subjects experienced urgency; and

The number of days per week that subjects experienced fecal incontinence.

Each of the secondary continuous variables will be analyzed as percent change from baseline.

The daily abdominal pain or discomfort score were assigned scores as follows: none=0, mild=1, moderate=2, severe=3, that is the greater the score the worse the pain or discomfort. Stool consistencies will be assigned scores as follows: 1=very hard, 2=hard, 3=formed, 4=loose, 5=watery for each bowel movement.

The stool consistency score was computed from the mean of these scores each day.

Table 1 below shows the baseline characteristics for the placebo-controlled treatment phase, including the diagnosed cause of diarrhea, the CD4 cell count and the CD4 cell category. This table demonstrates that the subjects in each group were similar.

TABLE 1

Baseline Characteristics in the Placebo-Controlled Treatment Phase					
Baseline Disease Characteristics	Placebo (N = 138)	Crofelemer 125 mg* (N = 138)	Crofelemer 250 mg (N = 54)	Crofelemer 500 mg (N = 47)	All Crofelemer (N = 239)
Cause of diarrhea					
Antiretroviral therapy	104 (75.4%)	104 (75.4%)	37 (68.5%)	30 (63.8%)	171 (71.5%)
HIV infection of intestine	33 (23.9%)	32 (23.2%)	15 (27.8%)	15 (31.9%)	62 (25.9%)
Other	1 (0.7%)	2 (1.4%)	2 (3.7%)	2 (4.3%)	6 (2.5%)
CD4 cell counts					
n	138	137	54	46	237
Mean	530.5	497.8	425.2	481.7	478.1
SD	244.79	230.88	226.13	275.18	239.81

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TABLE 1-continued

Baseline Characteristics in the Placebo-Controlled Treatment Phase					
Baseline Disease Characteristics	Placebo (N = 138)	Crofelmer 125 mg* (N = 138)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 47)	All Crofelmer (N = 239)
Median	518.5	479.0	374.0	421.5	429.0
Min	76	111	100	149	100
Max	1298	1183	1095	1734	1734
CD4 cell Category					
<404	39 (28.3%)	55 (39.9%)	29 (53.7%)	21 (44.7%)	105 (3.9%)
≥404	99 (71.7%)	32 (59.4%)	25 (46.3%)	25 (53.2%)	132 (55.2%)

[1] Baseline was the average of daily data from the 7 days prior to first dose day of randomized study drug.
[2] Baseline was the average of daily stool consistency scores from the 7 days prior to first dose day of randomized study drug. The daily score = (1*# of very hard stools + 2*# of hard stools + 3*# of formed stools + 4*# of loose stools + 5*# of watery stools)/(# of total stools).
[3] Baseline was the average of daily scores from the 7 days prior to first dose day of randomized study drug, none = 0, mild = 1, moderate = 2, severe = 3.
[4] Baseline = 7*A/B, A = # of days with event during the 7 days prior to first dose day of randomized study drug, B = # of days with non-missing assessments.

Table 2 below shows additional baseline characteristics of the placebo-controlled treatment phase, including use of antibiotics during the study.

TABLE 2

Baseline Characteristics of the Placebo-Controlled Treatment Phase					
Baseline Disease Characteristics	Placebo (N = 138)	Crofelmer 125 mg* (N = 138)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 47)	All Crofelmer (N = 239)
Use of a new antibiotic regimen during placebo-controlled treatment phase					
Yes	14 (10.1%)	9 (6.5%)	2 (3.7%)	1 (2.1%)	12 (5.0%)
No	124 (89.9%)	129 (92.5%)	52 (96.3%)	46 (97.9%)	227 (95.0%)

Tables 3 and 3a below show the percentage of subjects with clinical response, e.g., improvement of watery diarrhea, in the placebo-controlled treatment phase, and the change in response from baseline as a function of time, respectively. As can be seen from Table 3, all three treatment groups from Stage I were statistically significant for treatment of watery diarrhea, as well as the combined group of subjects dosed 125 mg in both stages. Table 3a sets forth data indicating that,

regardless of treatment group, the primary endpoint (Clinical Response) demonstrated responsiveness by consistently correlating with other daily assessments collected in the study for changes in symptoms scoring. Responders, i.e., subjects with ≤2 watery stools per week, had significantly greater improvements in daily symptom severity scores than non-responders at each week during the course of the study.

TABLE 3

Primary Efficacy Endpoint: Percentage of Subjects with Clinical Response in the Placebo-Controlled Treatment Phase				
	Placebo (N = 138)	Crofelmer 125 mg* (N = 136)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 46)
Stage I				
Responder-n/Ni (%)	1/50 (2.0%)	9/44 (20.5%)	5/54 (9.3%)	9/46 (19.6%)
Treatment Difference (vs. Placebo)		18.5%	7.3%	17.6%
1-Sided 97.5% CI (1)		(6.0%, ∞)	(-1.7%, ∞)	(5.3%, ∞)
1-Sided P-value (vs. Placebo) [1]		0.0019	0.0563	0.0024
Combined				
Responder-n/Ni (%)	11/138 (8.0%)	24/136 (17.6%)		
Treatment Difference (vs. Placebo)		9.6%		
1-Sided 97.5% CI (1)		(1.2%, ∞)		
1-Sided P-value (vs. Placebo) [1]		0.0096		

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TABLE 3a

Responsiveness of the Primary Endpoint (Clinical Response) -					
Week	Daily Question	Clinical Response ^a : Weekly Responder Mean Change from Baseline (±SD)	Clinical Response ^a : Weekly Non-Responder Mean Change from Baseline (±SD)	Difference Responder - Non-Responder	p-value
Week 1					
	Daily Watery Stools	-1.75 (0.901)	-0.51 (1.296)	-1.24	<0.0001
	Daily Stool Consistency ^b	-1.08 (0.589)	-0.19 (0.381)	-0.90	<0.0001
	Daily Abdominal Pain ^c	-0.41 (0.651)	-0.11 (0.493)	-0.30	0.0217
	Urgency ^d	-2.97 (2.299)	-0.75 (1.908)	-2.22	<0.0001
	Fecal Incontinence ^e	-1.48 (2.007)	-0.43 (1.867)	-1.06	0.0144
	Daily Stool Frequency	-1.13 (1.299)	-0.31 (1.810)	-0.83	<0.0001
Week 2					
	Daily Watery Stools	-2.03 (1.184)	-0.53 (1.392)	-1.50	<0.0001
	Daily Stool Consistency ^b	-1.14 (0.672)	-0.17 (0.379)	-0.97	<0.0001
	Daily Abdominal Pain ^c	-0.61 (0.701)	-0.12 (0.494)	-0.49	<0.0001
	Urgency ^d	-2.79 (2.418)	-0.79 (2.014)	-2.00	<0.0001
	Fecal Incontinence ^e	-1.71 (2.071)	-0.44 (1.982)	-1.27	<0.0001
	Daily Stool Frequency	-1.18 (1.331)	-0.34 (1.851)	-0.83	<0.0001
Week 3					
	Daily Watery Stools	-2.00 (1.280)	-0.65 (1.408)	-1.35	<0.0001
	Daily Stool Consistency ^b	-1.20 (0.734)	-0.22 (0.382)	-0.98	<0.0001
	Daily Abdominal Pain ^c	-0.51 (0.668)	-0.17 (0.517)	-0.34	0.0016
	Urgency ^d	-3.41 (2.215)	-0.98 (2.253)	-2.43	<0.0001
	Fecal Incontinence ^e	-1.65 (2.228)	-0.64 (2.121)	-1.01	0.0015
	Daily Stool Frequency	-1.20 (1.314)	-0.41 (1.992)	-0.79	<0.0001
Week 4					
	Daily Watery Stools	-1.89 (1.058)	-0.68 (1.430)	-1.21	<0.0001
	Daily Stool Consistency ^b	-1.07 (0.645)	-0.24 (0.410)	-0.83	<0.0001
	Daily Abdominal Pain ^c	-0.48 (0.658)	-0.19 (0.533)	-0.28	0.0058
	Urgency ^d	-3.11 (2.411)	-0.99 (2.166)	-2.12	<0.0001
	Fecal Incontinence ^e	-1.75 (2.140)	-0.57 (2.085)	-1.18	<0.0001
	Daily Stool Frequency	-1.07 (1.086)	-0.43 (2.039)	-0.64	<0.0001

^aClinical response in a week was defined as ≥ 2 watery stools during a given week.
^bStool consistency response was defined as < 4 daily stool consistency score during a given week
^cAbdominal pain and discomfort score: 0 = none, 1 = mild, 2 = moderate, and 3 = severe.
^dNumber of days per week with urgency = $7 \times A/B$, where A = # of days with urgency in the week, and B = # of days with assessments in the week.
^eNumber of days per week with fecal incontinence = $7 \times A/B$, where A = # of days with fecal incontinence in the week, and B = # of days with assessments in the week.
^fP-values were obtained from a Wilcoxon rank-sum test for comparison of the responder vs. non-responder groups.

Table 4 below shows the number of weeks of clinical response of subjects in the study. As shown below, the subjects dosed with 500 mg BID had more weeks of response to the treatment.

TABLE 4

Number of Weeks with Clinical Response in the Placebo-Controlled Treatment Phase				
Number of Weeks Responded	Placebo (N = 138)	Crofelmer 125 mg* (N = 136)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 46)
Stage I				
Number of Subjects	N = 50	N = 44	N = 54	N = 46
0 week	43 (86.0%)	32 (72.7%)	41 (75.9%)	25 (60.9%)
1 week	6 (12.0%)	2 (4.5%)	8 (14.8%)	9 (19.6%)
2 weeks		2 (4.5%)	2 (3.7%)	4 (8.7%)
3 weeks		4 (9.1%)	2 (3.7%)	4 (8.7%)
4 weeks	1 (2.0%)	3 (6.8%)	1 (1.9%)	1 (2.2%)

TABLE 4-continued

Number of Weeks with Clinical Response in the Placebo-Controlled Treatment Phase				
Number of Weeks Responded	Placebo (N = 138)	Crofelmer 125 mg* (N = 136)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 46)
Crofelemer vs. Placebo [1]				
Odds Ratio		2.41	2.02	4.21
95% CI		(0.84, 6.87)	(0.74, 5.56)	(1.56, 11.33)
p-value		0.1011	0.1727	0.0045

Table 5 below shows subjects with clinical response by month. As can be seen from the Table, responding subjects and response rate increases the longer a subject was administered crofelemer.

TABLE 5

Subjects with Monthly Clinical Response by Month in the Placebo-Free Extension Phase				
Responders in Placebo-free Extension Phase	Crofelmer 125 mg* (N = 219) n (%)	Crofelmer 250 mg (N = 68) n (%)	Crofelmer 500 mg (N = 50) n (%)	All Crofelemer (N = 337) n (%)
Month 1	87/218 (39.9%)	19/68 (27.9%)	14/50 (28.0%)	120/336 (35.7%)
Month 2	99/208 (47.6%)	31/60 (51.7%)	19/48 (39.6%)	149/316 (47.2%)
Month 3	111/198 (56.1%)	30/58 (51.7%)	17/47 (36.2%)	158/303 (52.1%)
Month 4	99/178 (55.6%)	27/54 (50.0%)	20/44 (45.5%)	146/276 (52.9%)
Month 5	90/155 (58.1%)	30/53 (56.6%)	17/42 (40.5%)	137/250 (54.8%)

Table 6 below shows the percent of subjects with stool consistency response to crofelemer. As can be seen from Table 6, subjects administered 500 mg and 250 mg BID responded better than did the 125 mg BID or the 250 mg BID.

TABLE 6

Percentage of Subjects with Stool Consistency Response in the Placebo-Controlled Treatment Phase				
	Placebo (N = 138)	Crofelmer 125 mg* (N = 136)	Crofelmer 250 mg (N = 54)	Crofelmer 500 mg (N = 46)
Stage I				
Responder-n/Ni (%)	11/50 (22.0%)	14/44 (31.8%)	20/54 (37.0%)	23/46 (50.0%)
Treatment Difference vs. Placebo		9.8%	15.0%	28.0%
1-Sided 97.5% CI [1]		(-8.1%, ⁰⁰)	(-2.6%, ⁰⁰)	(-8.8%, ⁰⁰)
1-Sided P-value (vs. Placebo) [1]		0.1412	0.0470	0.0021
Combined				
Responder-n/Ni (%)	49/186 (35.5%)	53/136 (39.0%)		
Treatment Difference vs. Placebo		3.5%		
1-Sided 97.5% CI [1]		[-5.0%, ⁰⁰)		
1-Sided P-value (vs. Placebo) [1]		0.1428		

Table 7 below shows the number of months of clinical response by subjects on crofelemer in the study. Table 7 again demonstrates that the longer the treatment, the better the response to treatment.

TABLE 7

Number of Months with Clinical Response in the Placebo-Free Extension Phase				
Number of Weeks Responded	Crofelmer 125 mg* (N = 219)	Crofelmer 250 mg (N = 66)	Crofelmer 500 mg (N = 50)	
0 month	69 (31.5%)	25 (36.8%)	25 (50.0%)	
1 month	31 (14.2%)	6 (8.8%)	4 (8.0%)	
2 months	19 (8.7%)	10 (14.7%)	4 (8.0%)	
3 months	29 (13.2%)	7 (10.3%)	3 (6.0%)	
4 months	25 (11.4%)	10 (14.7%)	4 (8.0%)	
5 months	46 (21.0%)	10 (14.7%)	10 (20.0%)	

Table 8 below shows the percentage of Caucasian and Hispanic subjects and all other races with clinical response. This table demonstrates that the Caucasian and Hispanic populations receiving crofelemer responded well to treatment.

TABLE 8

Percentage of Subjects with Clinical Response by Race in the Placebo-Controlled Treatment Phase				
	Placebo (N = 83)	Crofelmer 125 mg* (N = 84)	Crofelmer 250 mg (N = 44)	Crofelmer 500 mg (N = 38)
Stage I				
Responder - n/Ni (%)	0/36 (0.0%)	7/31 (22.6%)	3/44 (6.8%)	8/38 (21.1%)
Treatment Difference vs. Placebo		22.6%	6.5%	21.1%
[95% CI]		[7.9%, 37.3%]	[-0.6%, 14.3%]	[8.14%, 34.0%]
P-value vs. Placebo [1]		0.0030	0.2481	0.0053
Combined				
Responder - n/Ni (%)	6/83 (7.2%)	19/84 (22.6%)		
Treatment Difference vs. Placebo		15.4%		
[95% CI]		[4.9%, 25.9%]		
P-value (vs. Placebo) [1]		0.0083		

TABLE 8-continued

Percentage of Subjects with Clinical Response by Race in the Placebo-Controlled Treatment Phase				
	Placebo (N = 83)	Crofelmer 125 mg* (N = 84)	Crofelmer 250 mg (N = 44)	Crofelmer 500 mg (N = 38)
Race: White/Hispanic				
Stage I				
Responder - n/Ni (%)	1/14 (7.1%)	2/13 (15.4%)	2/10 (20.0%)	1/8 (12.5%)
Treatment Difference vs. Placebo [95% CI]		8.2% [-15.6%, 32.0%]	12.9% [-15.4%, 41.1%]	5.4% [-21.2%, 32.0%]
P-value vs. Placebo [1] Combined		0.5956	0.5504	1.0000
Stage 2				
Responder - n/Ni (%)	5/55 (9.1%)	5/52 (9.6%)		
Treatment Difference vs. Placebo [95% CI]		0.5% [-10.5%, 11.6%]		
P-value (vs. Placebo) [1]		1.0000		

Note:
Clinical response was defined as ≤ 2 watery stools per week during at least 2 of the 4 efficacy assessment weeks.
Note:
Percentage is based on Ni, number of subjects entered the stage or combine.

In another study, 400 subjects with chronic HIV associated diarrhea were treated with crofelemer or placebo for 7 days in an inpatient setting. Crofelemer was given at doses of 250 mg and 500 mg enteric-coated tablets or 500 mg enteric-coated beads four times daily, compared to a matching placebo. Subjects who responded to treatment were continued in a three-week blinded outpatient phase. The difference in decreased stool weight between subjects receiving crofelemer and subjects receiving placebo was not statistically significant by the primary efficacy analysis.

However, reanalysis of these data revealed that approximately 50% of the study population did not have watery diarrhea at study entry. Evaluation of the population with watery diarrhea and urgency at baseline revealed statistically significant improvement in stool frequency and weight ($p < 0.05$) with treatment. Changes in abnormal (watery and loose) stools were even greater, with significant ($p < 0.015$) improvements in abnormal stool weight and frequency observed at Day 7. Approximately 3 days were necessary for the anti-diarrheal effects of crofelemer to stabilize.

Table 9 below shows additional supportive data showing that crofelemer 125 mg, 250 mg and 500 mg were efficacious at treating HIV associated diarrhea.

TABLE 9

Primary Efficacy Endpoint Percentage of Subjects with Clinical Response*				
	Placebo (N = 138)	Crofelemer 125 mg (N = 136)	Crofelemer 250 mg (N = 54)	Crofelemer 500 mg (N = 46)
Stage 1				
Responder	1/50 (2.0%)	9/44 (20.5%)	5/54 (9.3%)	9/46 (19.6%)
Treatment Difference		18.50%	7.30%	17.60%

TABLE 9-continued

Primary Efficacy Endpoint Percentage of Subjects with Clinical Response*				
	Placebo (N = 138)	Crofelemer 125 mg (N = 136)	Crofelemer 250 mg (N = 54)	Crofelemer 500 mg (N = 46)
Stage 2				
Responder	10/88 (11.4%)	15/92 (16.3%)		
Treatment Difference Combined		4.90%		
Responder	11/138 (8.0%)	24/136 (17.6%)		
Treatment Difference		9.60%		
1-Sided 97.5% CI		[1.2%, ∞)		
1-Sided P-value (vs. Placebo)		0.0096		

* ≤ 2 watery BM/wk during ≥ 2 of 4 wks of the 4-wk placebo controlled period

Table 10 below shows that crofelemer is particularly efficacious in treating males with HIV associated diarrhea. Additional subgroup analysis is shown in FIG. 1. The primary endpoint was analyzed in subgroups defined by demographics and baseline characteristics to assess the consistency of the treatment effect. FIG. 1 provides a summary of each of these subgroup analyses, showing the treatment difference in percentage of responders (crofelemer 125 mg BID vs. placebo) with associated confidence intervals and p-values. As shown in the figure, consistent efficacy was observed across subgroups; a higher percentage of subjects treated with crofelemer 125 mg BID experienced clinical response compared with placebo in all subgroups analyzed.

TABLE 10

Effects Across Demographic & Baseline Characteristics-Gender*		
	Placebo (N = 138)	Crofelemer 125 mg (N = 136)
Gender: Male		
Responder - n/Ni (%)	9/116 (7.8%)	22/115 (19.1%)
Treatment Difference vs Placebo [95% CI]		11.40% [2.7%, 20.1%]
P-value vs Placebo		0.0124
Gender: Female		
Responder - n/Ni (%)	2/22 (9.1%)	2/21 (9.5%)
Treatment Difference vs Placebo [95% CI]		0.40% [-16.9%, 17.8%]
P-value vs Placebo		1

*Clinical response was defined as ≤ 2 watery stools per week during at least 2 of the 4 efficacy assessment weeks. Intent-to-Treat Population.

According to one aspect, provided herein are methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in a male HIV positive subject, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a male subject in need thereof.

Below, Table 11 shows that subject taking protease inhibitors responded particularly well to treatment with crofelemer.

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TABLE 11

Effects Across Demographic & Baseline Characteristics- Prior use of Protease Inhibitors*		
	Placebo (N = 138)	Crofelemer 125 mg (N = 136)
Use of PI at Screening - Yes:		
Responder - n/Ni (%)	6/97 (6.2%)	15/86 (17.4%)
Treatment Difference vs Placebo [95% CI]		11.30% [1.9%, 20.6%]
P-value vs Placebo		0.0204
Use of PI at Screening - No:		
Responder - n/Ni (%)	5/41 (12.2%)	9/50 (18.0%)
Treatment Difference vs Placebo [95% CI]		5.80% [-8.8%, 20.4%]
P-value vs Placebo		0.5638

*Clinical response was defined as <=2 watery stools per week during at least 2 of the 4 efficacy assessment weeks. Intent-to-Treat Population

According to one aspect, provided herein are methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject that has previously used protease inhibitors, comprising: administering about 250 mg to about 1000 mg per day; administering about 250 mg per day; administering about 500 mg per day; administering about 1000 mg per day; administering about 125 mg two times per day; administering about 250 mg two times per day; or administering about 500 mg two times per day of crofelemer to a male subject in need thereof. As used herein, "previously used" includes, for example, subjects who have used protease inhibitors (PIs) prior to crofelemer therapy or overlapping with crofelemer therapy, but the PI use began prior to the first dose of crofelemer therapy.

TABLE 12

Secondary Efficacy Endpoints	
Intent-to-Treat Population	
Watery BMs/day	No treatment differences between 125 mg vs. Pbo Stage 1: numerically greater in 500 mg vs. Pbo (-0.91/day vs. -0.62/day; p = 0.0713)
Stool consistency score	Significant improvement for 125 mg vs. Pbo (-0.35 vs. -0.25; p = 0.0168)
Daily abd pain/discomfort	No treatment differences between 125 mg vs. Pbo Stage 1: 125 mg vs. Pbo (-0.35 vs. -0.13; p = 0.0170)
Days/wk with urgency	No treatment differences between 125 vs. Pbo
Days/wk with fecal incontinence	Numerical differences for 125 mg vs. Pbo (-0.96 vs. -0.55; p = 0.0643)
BM/day	No treatment differences between 125 mg vs. Pbo

FIG. 1 and Table 13 show subjects with clinical response in the crossover to placebo-free phase of the safety population. PC=Placebo-controlled Phase and PF=Placebo-free Phase. This data shows that subjects previously on placebo had a sharp increase in efficacy when they were crossed-over onto 125 mg crofelemer. This data also demonstrates that crofelemer efficacy continued to rise with length of use.

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TABLE 13

Subjects with Clinical Response Crossover to Placebo-Free Phase, Safety Population					
Month	Statistic ¹	Placebo (N = 126)	Crofelemer 125 mg (N = 99)	Crofelemer 250 mg (N = 15)	Crofelemer 500 mg (N = 12)
Month 1	Responder - n/Ni (%)	11/126 (8.7%)	36/99 (36.4%)	3/15 (20.0%)	2/12 (16.7%)
	Odds Ratio (95% CI)		0.17 (0.09, 0.32)		
	P-value (vs. Placebo in PC)		<.0001		
Month 2	Responder - n/Ni (%)		42/95 (44.2%)	8/15 (53.3%)	2/12 (16.7%)
	Odds Ratio (95% CI)		0.12 (0.06, 0.24)		
	P-value (vs. Placebo in PC)		<.0001		
Month 3	Responder - n/Ni (%)		48/89 (53.9%)	5/14 (35.7%)	2/11 (18.2%)
	Odds Ratio (95% CI)		0.07 (0.03, 0.15)		
	P-value (vs. Placebo in PC)		<.0001		
Month 4	Responder - n/Ni (%)		43/77 (55.8%)	5/13 (38.5%)	2/9 (22.2%)
	Odds Ratio (95% CI)		0.07 (0.03, 0.15)		
	P-value (vs. Placebo in PC)		<.0001		
Month 5	Responder - n/Ni (%)		37/67 (55.2%)	6/13 (46.2%)	1/8 (12.5%)
	Odds Ratio (95% CI)		0.07 (0.03, 0.16)		
	P-value (vs. Placebo in PC)		<.0001		

¹ Ratio of responders and p value obtained from parameter estimates with effect for treatment and region.

TABLE 14

Subjects with Stool Consistency Response* Intent-to-Treat Population				
	Placebo (N = 138)	Crofelemer 125 mg (N = 136)	Crofelemer 250 mg (N = 54)	Crofelemer 500 mg (N = 46)
Stage 1				
45 Responder	11/50 (22.0%)	13/44 (31.8%)	20/54 (37.0%)	23/46 (50.0%)
Treatment Difference				
Stage 2				
50 Responder	38/88 (43.2%)	39/92 (42.4%)		
Treatment Difference Combined				
55 Responder	49/138 (35.5%)	53/136 (39.0%)		
Treatment Difference				

*<4stool consistency score at least 2 of the 4 efficacy assessment weeks.

FIG. 2 and Table 14 show subjects with stool consistency response in the crossover to placebo-free phase in the safety population. PC=Placebo-controlled Phase and PF=Placebo-free Phase. This data shows that subjects previously on placebo had a sharp increase in efficacy when they were crossed-over onto 125 mg crofelemer. This data also demonstrates that crofelemer efficacy continued to rise with length of use.

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TABLE 15

Subjects With Abnormal ECG Findings Safety Population					
Characteristic, n (%)	Placebo (N = 137)	Crofelemer 125 mg (N = 130)	Crofelemer 250 mg (N = 54)	Crofelemer 500 mg (N = 42)	All Crofelemer (N = 226)
Post Baseline Abnormal ECG Finding ¹	35 (25.5%)	34 (26.2%)	18 (33.3%)	13 (31.0%)	65 (28.8%)
QT Interval (msec)					
>450 with Baseline ≤ 450	2 (1.5%)	4 (3.1%)	1 (1.9%)	0	5 (2.2%)
>480 with Baseline ≤ 480	1 (0.7%)	0	1 (1.9%)	0	1 (0.4%)
>500 with Baseline ≤ 500	0	0	1 (1.9%)	0	1 (0.4%)
Change from Baseline 30-60	9 (6.6%)	4 (3.1%)	6 (11.1%)	3 (7.1%)	13 (5.8%)
Change from Baseline > 60	4 (2.9%)	1 (0.8%)	2 (3.7%)	0	3 (1.3%)
QT Interval Linear Regression Correction (msec)					
>450 with Baseline ≤ 450	2 (1.5%)	4 (3.1%)	1 (1.9%)	0	5 (2.2%)
>480 with Baseline ≤ 480	1 (0.7%)	0	1 (1.9%)	0	1 (0.4%)
>500 with Baseline ≤ 500	0	0	1 (1.9%)	0	1 (0.4%)
Change from Baseline 30-60	7 (5.1%)	4 (3.1%)	6 (11.1%)	3 (7.1%)	13 (5.8%)
Change from Baseline > 60	4 (2.9%)	1 (0.8%)	2 (3.7%)	0	3 (1.3%)
QT Interval Fridericias's Correction (msec)					
>450 with Baseline ≤ 450	6 (4.4%)	1 (0.8%)	2 (3.7%)	0	3 (1.3%)
>480 with Baseline ≤ 480	1 (0.7%)	1 (0.8%)	0	0	1 (0.4%)
>500 with Baseline ≤ 500	1 (0.7%)	0	0	0	0
Change from Baseline 30-60	3 (2.2%)	5 (3.8%)	2 (3.7%)	1 (2.4%)	8 (3.5%)
Change from Baseline > 60	4 (2.9%)	0	1 (1.9%)	0	1 (0.4%)
QT Interval Bazett's Correction (msec)					
>450 with Baseline ≤ 450	8 (5.8%)	4 (3.1%)	2 (3.7%)	0	6 (2.7%)
>480 with Baseline ≤ 480	2 (1.5%)	2 (1.5%)	1 (1.9%)	0	3 (1.3%)
>500 with Baseline ≤ 500	2 (1.5%)	0	0	0	0
Change from Baseline 30-60	7 (5.1%)	8 (6.2%)	3 (5.6%)	1 (2.4%)	12 (5.3%)
Change from Baseline > 60	3 (2.2%)	1 (0.8%)	1 (1.9%)	0	2 (0.9%)

Table 15 demonstrates that crofelemer is safe to be given to subjects and that there are no QT Interval issues associated with use. This is surprising and advantageous over other molecules used to treat diarrhea and IBS, which are known to be associated with QT issues.

From the above data and Figures, it has been demonstrated that the proportion of clinical responders was significantly higher in the crofelemer 125 mg group compared with placebo (p=0.0096; Combined data). Subjects switching from placebo to crofelemer upon conclusion of the placebo-controlled phase achieved rates of clinical response of between 36.4% and 55.8% for each of the 5 months of the placebo-free phase (p<0.0001; for data collected prior to lock). Stool consistency scores improved significantly in subjects receiving 125 mg crofelemer compared with placebo (p=0.0168; Combined data). Days per week that subjects experienced fecal incontinence decreased in subjects receiving 125 mg crofelemer compared with placebo (p=0.0643; Combined data). It is also demonstrated that crofelemer was well tolerated and demonstrated a safety profile comparable to placebo and no clinically important differences in safety assessments have been identified.

TABLE 16

Dose-Ranging Study for treatment of d-IBS: All Randomized Subjects	
Design:	P2, R, DB, PC, Dose-Ranging Study for treatment of d-IBS
Treatment:	125, 250, 500 mg Crofelemer b.i.d. or Pbo for 12 weeks
N:	125 mg: 62, 250 mg: 60, 250 mg: 62, Pbo: 61
Primary Endpoint	Daily stool consistency
Outcome:	ROME Foundation Definition ¹ Month 1 = 2.6%
	Treatment Δ, 125 mg dose Month 2 = 9.0%
	Month 3 = 10.7%
	All 3 months: Odds Ratio 1.51 (0.78, 2.92)
	FDA Definition ² Month 1 = 0.6%
Outcome:	Treatment Δ, 125 mg dose Month 2 = 2.2%
	Month 3 = -4.4%
	All 3 months: Odds Ratio 1.09 (0.54, 2.20)

¹ROME Foundation Stool consistency weekly responder: Subjects with <25% days with loose or watery stools in a given week. Based on Ad Hoc Table 1.1
²FDA Stool consistency weekly responder: Subjects with a weekly average stool consistency score <4 (4 = Loose stool)

Table 16 demonstrates that crofelemer is an efficacious treatment for d-IBS. It also demonstrates that crofelemer is an efficacious treatment for treating abnormal stool consistency associated with d-IBS.

In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS for at least one month.

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In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS from between one month and two months or longer. In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS from between about one month and about three months or longer.

In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS for at least one month at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS from between one month and two months or longer at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS from between one month and three months or longer at about 125 mg b.i.d.

TABLE 17

Dose-Ranging Study for treatment of d-IBS All Randomized Female Subjects		
Design:	P2, R, DB, PC, Dose-Ranging Study for treatment of d-IBS	
Treatment:	125, 250, 500 mg Crofelemer b.i.d. or Pbo for 12 weeks	
N (125 mg females):	125 mg: 46, Pbo: 46	
Primary Endpoint	Daily stool consistency	
Outcome:	ROME Foundation Definition ¹	
	Treatment Δ, 125 mg dose	Month 1 = 6.5% Month 2 = 10.8% Month 3 = 10.8%
	All 3 months: Odds Ratio	1.88 (0.87, 4.06)
	FDA Definition ²	Month 1 = -2.1% Month 2 = 2.2% Month 3 = -4.4%
	Treatment Δ, 125 mg dose	
	All 3 months: Odds Ratio	1.20 (0.52, 2.75)

¹ROME Foundation Stool consistency weekly responder: Subjects with <25% days with loose or watery stools in a given week. Based on Ad Hoc Table 1.1.
²FDA Stool consistency weekly responder: Subjects with a weekly average stool consistency score <4 (4 = Loose stool)

Table 17 demonstrates that crofelemer is an efficacious treatment for d-IBS, especially to treat d-IBS in females. It also demonstrates that crofelemer is an efficacious treatment for treating abnormal stool consistency associated with d-IBS, and especially to treat abnormal stool consistency associated with d-IBS in females.

In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS in females for at least one month at 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS in females from between one month and two months at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abnormal stool consistency associated with d-IBS in females from between one month and three months or longer at about 125 mg b.i.d.

TABLE 18

Dose-Ranging Study for treatment of d-IBS in females All Randomized Subjects			
Design:	P2, R, DB, PC, Dose-Ranging Study for treatment of d-IBS in females		
Treatment:	125 mg Crofelemer b.i.d. or Pbo for 12 weeks		
N:	125 mg: 120, Pbo: 120		
Primary Endpoint	Daily abdominal pain ¹		
Outcome:	Placebo (N = 120)	125 mg (N = 120)	P-value
Month 1	66 (55.0%)	75 (62.5%)	0.2316
Month 2	61 (50.8%)	82 (68.3%)	0.0059
Month 3	65 (54.2%)	79 (65.8%)	0.0662
All 3 Months	Odds Ratio: 1.67 (1.03, 2.70); p = 0.0357		

¹Abdominal pain weekly responder is defined as subjects with at least 30% improvement compared to baseline abdominal pain score in a given week

Table 18 demonstrates that crofelemer is an efficacious treatment for d-IBS, especially to treat d-IBS in females. It also demonstrates that crofelemer is an efficacious treatment for treating abdominal pain associated with d-IBS, and especially to treat abdominal pain associated with d-IBS in females.

In one embodiment, crofelemer is administered to treat d-IBS for at least one month.

In one embodiment, crofelemer is administered to treat d-IBS from between one month and two months or longer. In one embodiment, crofelemer is administered to treat d-IBS from between about one month and about three months or longer.

In one embodiment, crofelemer is administered to treat d-IBS for at least one month at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat d-IBS from between one month and two months or longer at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat d-IBS from between one month and three months or longer at about 125 mg b.i.d.

In one embodiment, crofelemer is administered to treat d-IBS in females for at least one month at 125 mg b.i.d. In one embodiment, crofelemer is administered to treat d-IBS in females from between one month and two months at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat d-IBS in females from between one month and three months or longer at about 125 mg b.i.d.

In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS for at least one month.

In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS from between one month and two months or longer. In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS from between one month and three months or longer at about 125 mg b.i.d.

In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS in females for at least one month at 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS in females from between one month and two months at about 125 mg b.i.d. In one embodiment, crofelemer is administered to treat abdominal pain associated with d-IBS in females from between one month and three months or longer at about 125 mg b.i.d.

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to treat abdominal pain associated with d-IBS in females from between one month and three months or longer at about 125 mg b.i.d.

Example 10

A Double-Blind, Randomized, Placebo-Controlled, Phase II Study to Assess the Safety and Efficacy of Orally Administered Crofelemer for the Symptomatic Treatment of Diarrhea in Acquired Immunodeficiency Syndrome (AIDS) Patients

This study was a randomized, double-blind, multicenter (2 study sites), placebo-controlled, parallel-group study designed to assess the efficacy and safety of crofelemer 500 mg beads in subjects with HIV-associated diarrhea.

The primary objectives of the study were to evaluate the safety and efficacy of orally administered crofelemer for 96 hours for the symptomatic treatment of diarrhea in AIDS patients. The secondary objectives were 1) to characterize stool chloride ion concentration and daily stool chloride output in AIDS patients with diarrhea, 2) to compare stool chloride ion concentration and daily stool chloride output in AIDS patients with diarrhea treated with crofelemer or placebo, and 3) to assess stool consistency in AIDS patients with diarrhea treated with crofelemer or placebo.

There were 3 assessment periods during the study: 1) a 24-hour in-patient screening period to ensure that the subjects met all of the study criteria, during which baseline stool weight was assessed; 2) a 4-day inpatient treatment period, during which all subjects received their assigned treatment 4 times per day (Days 1-4); subjects were discharged from the hospital after 96 hours of treatment if clinically stable; and 3) a follow-up visit 7-9 days after discharge from the hospital. The use of ADM was not allowed during the study.

Efficacy measurements included assessments of stool weight and frequency, abnormal stool frequency, DGIS, MORE, body weight, time to diarrhea recurrence, and number of early dropouts (prior to completion of 4 days of treatment).

Efficacy endpoints in this study were as follows:

The primary efficacy endpoint in this study was the change in total daily stool weight during the Treatment Period. The secondary efficacy endpoints of the study were abnormal stool frequency, defined as watery or soft stools (change in daily abnormal stool frequency), stool frequency (change in daily stool frequency), DGIS (change from baseline in DGIS for each day [Days 1 through 4]), stool chloride concentration (mg chloride/g stool weight; change in daily stool chloride concentration) and measure of relief scores, where MORE was the maximum of: a) time from the start of treatment period to the first abnormal stool, b) the maximum time between abnormal stools, or c) the time between the last abnormal stool and the end of the treatment period.

The DGIS was the daily sum of mean symptom scores for each of 7 symptoms (nausea, vomiting, abdominal pain and/or cramps, excess gas, urgency, tenesmus, and incontinence) scored 4 times per day. Symptoms were rated on a 4-point scale from 0=absent to 3=severe.

A total of 85 subjects were enrolled into the study at 2 study sites. Two of subjects in each of the 2 treatment groups withdrew before completion of the in-patient treatment period.

All randomized subjects (n=85) received at least 1 dose of study drug and were included in efficacy analyses.

TABLE 19

Demographics:		
Characteristic Category or Statistic	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)
Age, Years		
Mean (±SD)	38.9 (7.6)	41.0 (8.8)
Median	36	40
Min, Max	27, 55	21, 60
Sex, n (%)		
Male	41 (97.6)	42 (97.7)
Female	1 (2.4)	1 (2.3)
Race, n (%)		
Caucasian	26 (61.9)	32 (74.4)
Hispanic	7 (16.7)	6 (14.0)
African American	7 (16.7)	5 (11.6)
Other	2 (5.0)	0

Abbreviations:
ITT = intent-to-treat;
Max = maximum;
Min = minimum;
SD = standard deviation.

The number of unformed (i.e., soft or watery) stools/day at baseline (screening [Day 0]) and during the week prior to baseline, and disease severity are presented in Table. Mean (±SD) unformed stools/day at baseline was 5.5 (3.3) in the placebo group and 4.6 (2.6) in the crofelemer group. Most subjects had mild (3-4 stools/day) or moderate (5-8 stools/day) disease severity at baseline. Five subjects in the crofelemer group and 4 subjects in the placebo group had severe diarrhea (>9 stools/day).

TABLE 20

Baseline Diarrhea Assessments: (ITT Population)		
Characteristic Category or Statistic	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)
Unformed Stools During 24 Hours Prior to First Dose of Study Drug (Screening [Day 0]), Stools/Day		
Mean (±SD)	5.6 (2.9)	4.6 (2.5)
Median	5	4
Min, Max	1, 6	0, 10
Unformed Stools During the Week Prior to First Dose of Study Drug (Screening [Day 0]), Stools/Day		
Mean (±SD)	5.5 (2.9)	5.1 (2.3)
Median	6	5
Min, Max	1, 15	2, 10
Diarrhea Severity, n (%)		
Mild (3-4 stools/day)	10 (40.0)	15 (57.1)
Moderate (5-8 stools/day)	13 (52.0)	8 (30.8)
Severe (>9 stools/day)	2 (8.0)	3 (11.5)

Abbreviations:
ITT = intent-to-treat;
Max = maximum;
Min = minimum;
SD = standard deviation.

Concomitant antiretroviral medications were received by majority of subjects (71 of 85): 36 of 42 (85.7%) in the placebo group and 35 of 43 (81.4%) in the crofelemer group. Protease inhibitors were taken by 69.4% of subjects. The concomitant use of antiretrovirals, including protease inhibitors, was balanced between groups.

The primary efficacy analysis was change in total daily stool weight during the 4-day in-patient treatment period. The

primary endpoint of reduction in stool weight is an appropriate measure of the extent of watery diarrhea in patients with HIV-associated diarrhea due to high water content in the diarrhea experienced by these patients.

As shown in Table 21, there were significantly greater decreases in stool weight from baseline to Day 4 (last treatment day) in the crofelemer group compared with placebo (p=0.0335 by generalized linear model) in the ITT population. The repeated measures analysis of longitudinal data over the course of the 4-day Treatment Period did not show significant improvements in the crofelemer group compared to placebo; p=0.4108 for changes in total stool weight.

TABLE 21

Change in Stool Weight			
In-Patient Period	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)	p-value (vs. Placebo) ^a
Stool Weight at Baseline (g)			
Mean (±SD)	730.9 (720.14)	861.3 (604.67)	0.3832
Median	547.0	707.7	
Min, Max	206, 4701	220, 3407	
Categories of Stool Weight at Baseline, n (%)			
Low (<740 g)	28 (66.7)	24 (55.8)	0.2725
High (>740 g)	14 (33.3)	19 (44.2)	
Change in Stool Weight: Baseline to Day 4 (g)			
Mean (±SD)	-192.4 (381.57)	-401.3 (531.65)	0.0335
Median	-232.8	-267.5	
Min, Max	-1319, 683	-1815, 854	

Abbreviations:
ITT = intent-to-treat;
Max = maximum;
Min = minimum,
SD = standard deviation.

^aP-value for baseline mean comparison is from generalized linear model with analysis center as a covariate. P-value for baseline percentage comparison is from CMH test with analysis center as a covariate. The estimates and p values are from the generalized linear model for the change from baseline result, with independent variables: treatment, analysis center, baseline category (value = Low for ≤740 g and High for >740 g in stool weight), and the interaction between treatment and baseline category (if p value > 0.15, the interaction term was not included).

The effect of crofelemer in decreasing stool weight was more pronounced in the subgroup of subjects with baseline stool weight >740 g when compared to subjects with baseline stool weight ≤740 g (p-values for differences relative to placebo were 0.0202 [>740 g subgroup] versus 0.6820 [≤740 g subgroup]).

There were significantly greater decreases from baseline in stool weight at Day 3 in the crofelemer group compared with placebo (p=0.0128).

Secondary Efficacy Results

Significantly greater decreases in the frequency of abnormal stools (i.e., watery or soft stools) from baseline to Day 4 were observed in the crofelemer group compared with placebo (p=0.0069 by generalized-linear model) in the ITT population. The repeated measures analysis of longitudinal data over the course of the Treatment Period also indicates significantly greater reductions in the crofelemer group compared with placebo; p=0.0330 for changes in abnormal stool frequency. In addition, subjects in the crofelemer group had significantly greater decreases in abnormal stool frequency from baseline to Day 2 (p=0.0454) and from baseline to Day 3 (p=0.0064) compared with subjects in the placebo group.

TABLE 22

Abnormal Stool Frequency			
In-Patient Period	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)	p-value (vs. Placebo) ^a
Abnormal Stool (soft or watery stool) Frequency at Baseline; abnormal stools/day			
Mean (±SD)	4.8 (2.12)	4.9 (2.58)	0.9933
Median	4.0	4.0	
Min, Max	3, 12	2, 14	
Categories of Abnormal Stool Frequency at Baseline, n (%)			
Low (≤5/day)	30 (71.4)	32 (74.4)	0.7847
High (>5/day)	12 (28.6)	11 (25.6)	
Change in Abnormal Stool Frequency: Baseline to Day 4; abnormal stools/day			
Mean (±SD)	-2.1 (1.94)	-2.8 (2.23)	0.0069
Median	-2.0	-3.0	
Min, Max	-6, 4	-11, 2	

Abbreviations:
ITT = intent-to-treat;
Max = maximum;
Min = minimum,
SD = standard deviation.

^aP-value for baseline mean comparison is from generalized linear model with analysis center as a covariate. P-value for baseline percentage comparison is from CMH test with analysis center as a covariate. The estimates and p values are from the generalized linear model for the change from baseline result, with independent variables: treatment, analysis center, baseline category (value = Low for ≤5/day and High for >5/day in abnormal stool frequency), and the interaction between treatment and baseline category (if p value > 0.15, the interaction term was not included).

The effect of crofelemer in decreasing abnormal stool frequency was more pronounced in the subgroup with high abnormal stool counts at baseline (>5/day) compared to the subgroup with low abnormal stool counts at baseline (≤5/day; p-values for differences relative to placebo were 0.0041 [>5/day subgroup] versus 0.8184 [≤5/day subgroup]).

Significantly greater decreases in stool frequency (i.e., formed, watery, and soft stools) from baseline to Day 4 were observed in the crofelemer group compared with placebo (p=0.0046 by generalized-linear model) in the ITT population (Table 23). The repeated measures analysis of longitudinal data over the course of the Treatment Period also indicates significantly greater reductions in the crofelemer group compared with placebo; p=0.0236 for changes in stool frequency.

The effect of crofelemer in decreasing stool frequency was more pronounced in the subgroup with high stool counts at baseline (>5/day) compared to the subgroup with low stool counts at baseline (≤5/day) (p-values for differences relative to placebo were 0.0019 [>5/day subgroup] versus 0.7912 [≤5/day subgroup]).

TABLE 23

Stool Frequency			
In-Patient Period	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)	p-value (vs. Placebo) ^a
Stool Frequency at Baseline; stools/day			
Mean (±SD)	5.1 (2.22)	5.0 (2.46)	0.8442
Median	4.0	4.0	
Min, Max	3, 12	2, 14	
Categories of Stool Frequency at Baseline, n (%)			
Low (≤5/day)	27 (64.3)	32 (74.4)	0.2850
High (>5/day)	15 (35.7)	11 (25.6)	

TABLE 23-continued

In-Patient Period	Stool Frequency		
	Placebo (n = 42)	Crofelemer 500 mg Beads (n = 43)	p-value (vs. Placebo) ^a
Change in Stool Frequency: Baseline to Day 4; stools/day			
Mean (±SD)	-1.7 (1.92)	-2.5 (2.45)	0.0116
Median	-2.0	-2.5	
Min, Max	-5, 5	-10, 2	

Abbreviations:
ITT = intent-to-treat;
Max = maximum;
Min = minimum;
SD = standard deviation.
^aP-value for baseline mean comparison is from generalized linear model with analysis center as a covariate. P-value for baseline percentage comparison is from CMH test with analysis center as a covariate. The estimates and p values are from the generalized linear model for the change from baseline result, with independent variables: treatment, analysis center, baseline category (value = Low for ≤3/day and High for >3/day in stool frequency), and the interaction between treatment and baseline category (if p value >0.15, the interaction term was not included).

The crofelemer group had significantly greater decreases in stool frequency from baseline to Day 2 (p=0.0223) and from baseline to Day 3 (p=0.0140) compared with placebo.

Daily Gastrointestinal Symptom Score

In a repeated measures analysis of longitudinal data over the course of the in-patient period (i.e., changes from baseline at each day during Days 1-4), a statistical trend indicating greater improvements in DGIS scores was observed in the crofelemer group compared with placebo (p=0.0559).

Stool Chloride Concentrations

Stool chloride concentrations were measured in this study because the antisecretory, antidiarrheal effect of crofelemer is likely due to the inhibition of CFTR channel and CACC in the GI lumen; this inhibition blocks luminal Cl⁻ secretion and accompanying high volume water loss in secretory diarrhea (Fischer 2004; Tradtrantip 2010); thus reduced luminal Cl⁻ secretion should result in lower stool chloride concentrations. Subjects in the crofelemer group had significantly greater reductions in stool chloride concentrations from baseline to Day 4 when compared with placebo (p=0.0024 by generalized linear model) among subjects with stool chloride data (placebo n=25, crofelemer n=26). Mean (±SD) changes from baseline to Day 4 were 0.123 (0.7138) mg/g in the placebo group and -0.245 (0.5556) mg/g in the crofelemer group.

All publications, patents, and patent applications cited herein are hereby incorporated herein by reference in their entirety.

What is claimed is:

- 1. A method of treating stool consistency in an HIV positive subject, comprising:
administering about 250 mg of crofelemer per day to the HIV positive subject.
- 2. A method of improving stool consistency in an HIV positive subject, comprising:
administering about 250 mg of crofelemer per day to the HIV positive subject.
- 3. A method of alleviating watery diarrhea in an HIV positive subject, comprising:
administering about 250 mg of crofelemer per day to the HIV positive subject.
- 4. A method of decreasing the number of bowel movements per day in an HIV positive subject, comprising:
administering about 250 mg of crofelemer per day to the HIV positive subject.

5. The method of any one of claim 1, 2, 3 or 4 wherein the administering is from between about 1 month and about 6 months.

6. The method of any one of claim 1, 2, 3 or 4 wherein the administering is from between about 3 days and 6 months.

7. The method of any one of claim 1, 2, 3 or 4 wherein improvement of symptoms begins on day 3.

8. The method of any one of claim 1, 2, 3 or 4 wherein improvement of symptoms increases with a longer duration of administration after day 3.

9. The method of any one of claim 1, 2, 3 or 4 wherein the subject is of Caucasian or Hispanic descent.

10. The method of any one of claim 1, 2, 3 or 4 wherein the crofelemer is administered for at least 8 days.

11. The method of any one of claim 1, 2, 3 or 4 wherein the crofelemer is administered from between 8 days and 24 weeks.

12. The method of any one of claim 1, 2, 3 or 4 wherein the administering is for about 6 months.

13. The method of any one of claim 1, 2, 3 or 4 wherein the administering is about 6 months or longer.

14. The method of any one of claim 1, 2, 3 or 4 wherein the administering is for the duration of the HIV infection.

15. The method of any one of claim 1, 2, 3 or 4 wherein the response to treatment increases after the crofelemer has been administered for longer than 4 months.

16. The method of any one of claim 1, 2, 3 or 4 wherein the subject is considered treated if the subject demonstrates one or more of: a decrease in the number of bowel movements per day, a decrease in the number of watery bowel movements per day, an improvement in the daily abdominal score for pain or discomfort, an improvement in the score for daily stool consistency, a decrease in stool consistency, a decrease in the number of days per week that subjects experienced urgency, a decrease in the number of days per week that subjects experienced fecal incontinence, or a decrease in the unscheduled visit for a significant worsening of diarrhea.

17. The method of claim 1, wherein subject is considered treated if the subject demonstrates an improvement in the score for daily stool consistency.

18. The method of claim 2, wherein subject is considered treated if the subject demonstrates a decrease in stool consistency.

19. The method of claim 3, wherein subject is considered treated if the subject demonstrates a decrease in the number of watery bowel movements per day.

20. The method of claim 4, wherein subject is considered treated if the subject demonstrates a decrease in the number of bowel movements per day.

21. The method of claim 16, wherein symptoms increased or decreased are measured from a baseline.

22. A method of decreasing the number of days per week a subject experiences fecal incontinence associated with HIV associated diarrhea or HAART associated diarrhea, comprising: administering about 250 mg of crofelemer per day to the subject experiencing fecal incontinence associated with HIV or HAART.

23. The method of claim 1, comprising administering about 125 mg of crofelemer to the subject two times per day.

24. The method of claim 2, comprising administering about 125 mg of crofelemer to the subject two times per day.

25. The method of claim 3, comprising administering about 125 mg of crofelemer to the subject two times per day.

26. The method of claim 4, comprising administering about 125 mg of crofelemer to the subject two times per day.

27. The method of claim 22, comprising administering about 125 mg of crofelemer to the subject two times per day.

* * * * *

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APPENDIX B: ISSUED CLAIMS
U.S. Patent 8,962,680, Issued on February 24, 2015
Our Ref.: 119557-06006

1. A method of treating stool consistency in an HIV positive subject, comprising: administering about 250 mg of crofelemer per day to the HIV positive subject.
2. A method of improving stool consistency in an HIV positive subject, comprising: administering about 250 mg of crofelemer per day to the HIV positive subject.
3. A method of alleviating watery diarrhea in an HIV positive subject, comprising: administering about 250 mg of crofelemer per day to the HIV positive subject.
4. A method of decreasing the number of bowel movements per day in an HIV positive subject, comprising: administering about 250 mg of crofelemer per day to the HIV positive subject.
5. The method of any one of claim 1, 2, 3 or 4 wherein the administering is from between about 1 month and about 6 months.
6. The method of any one of claim 1, 2, 3 or 4 wherein the administering is from between about 3 days and 6 months.
7. The method of any one of claim 1, 2, 3 or 4 wherein improvement of symptoms begins on day 3.
8. The method of any one of claim 1, 2, 3 or 4 wherein improvement of symptoms increases with a longer duration of administration after day 3.
9. The method of any one of claim 1, 2, 3 or 4 wherein the subject is of Caucasian or Hispanic descent.
10. The method of any one of claim 1, 2, 3 or 4 wherein the crofelemer is administered for at least 8 days.
11. The method of any one of claim 1, 2, 3 or 4 wherein the crofelemer is administered from between 8 days and 24 weeks.
12. The method of any one of claim 1, 2, 3 or 4 wherein the administering is for about 6 months.
13. The method of any one of claim 1, 2, 3 or 4 wherein the administering is about 6 months or longer.
14. The method of any one of claim 1, 2, 3 or 4 wherein the administering is for the duration of the HIV infection.
15. The method of any one of claim 1, 2, 3 or 4 wherein the response to treatment increases after the crofelemer has been administered for longer than 4 months.
16. The method of any one of claim 1, 2, 3 or 4 wherein the subject is considered treated if the subject demonstrates one or more of: a decrease in the number of bowel movements per day, a decrease in the number of watery bowel movements per day, an improvement in the daily abdominal score for pain or discomfort, an improvement in the score for daily stool consistency, a decrease in stool consistency, a decrease in the number of days per week that subjects experienced urgency, a decrease in the number of days per week that subjects experienced fecal incontinence, or a decrease

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in the unscheduled visit for a significant worsening of diarrhea.

17. The method of claim 1, wherein subject is considered treated if the subject demonstrates an improvement in the score for daily stool consistency.

18. The method of claim 2, wherein subject is considered treated if the subject demonstrates a decrease in stool consistency.

19. The method of claim 3, wherein subject is considered treated if the subject demonstrates a decrease in the number of watery bowel movements per day.

20. The method of claim 4, wherein subject is considered treated if the subject demonstrates a decrease in the number of bowel movements per day.

21. The method of claim 16, wherein symptoms increased or decreased are measured from a baseline.

22. A method of decreasing the number of days per week a subject experiences fecal incontinence associated with HIV associated diarrhea or HAART associated diarrhea, comprising: administering about 250 mg of crofelemer per day to the subject experiencing fecal incontinence associated with HIV or HAART.

23. The method of claim 1, comprising administering about 125 mg of crofelemer to the subject two times per day.

24. The method of claim 2, comprising administering about 125 mg of crofelemer to the subject two times per day.

25. The method of claim 3, comprising administering about 125 mg of crofelemer to the subject two times per day.

26. The method of claim 4, comprising administering about 125 mg of crofelemer to the subject two times per day.

27. The method of claim 22, comprising administering about 125 mg of crofelemer to the subject two times per day.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: _____
Electronic Signature _____

Docket No.: 119557-06005

Docket No.: 119557-06005
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Utility Application of:
William Forbes *et al.*

Application No.: 13/294,307

Confirmation No.: 1066

Filed: November 11, 2011

Art Unit: 1615

For: METHODS AND COMPOSITIONS FOR
TREATING HIV-ASSOCIATED DIARRHEA

Examiner: W. A. Craig

DECLARATION OF HERBERT L. DUPONT UNDER 37 C.F.R. § 1.132

I, Herbert L. DuPont, M.D., declare and state the following:

1. I am the Chief of Internal Medicine at St. Luke's Hospital, as well as the Director of the Center for Infectious Diseases at the University of Texas – Houston School of Public Health, and the Vice Chairman of the Department of Medicine at Baylor College of Medicine. I also hold the Mary W. Kelsey Chair of Medical Sciences at The University of Texas – Houston, and I have served as an epidemic intelligence service officer at the Centers for Disease Control (CDC). I received my B.A. degree from Ohio Wesleyan University and my M.D. degree from Emory University School of Medicine. I completed an internal medicine internship and residency at the University of Minnesota and an infectious disease fellowship at the University of Maryland. I am licensed to practice in Texas and Georgia. My curriculum vitae is attached as Exhibit A.

2. I am a consultant for Salix Pharmaceuticals, one of the assignees of the instant application. Salix will not provide me with any special or additional compensation in exchange for this declaration, and I have no direct financial interest in the outcome of this application.

3. I have studied the claims that are pending in the instant application, which are directed to methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject by administering a total of about 250 mg of crofelemer per day to a subject in need thereof.

4. I have also studied the Office Action dated March 11, 2013, and the Holodniy (*Am. J. Gastroenterol.*, 1999), McMahan (*Alimentary Pharmacol. & Therapeutics*, 2007)¹ and Quart (U.S. Patent Publication No. 2007/0254050) references cited in the rejection of the claims as being obvious.

5. The obviousness rejection appears to be based on the grounds that, although diarrhea-predominant irritable bowel syndrome ("d-IBS") is a different disease than diarrhea associated with HIV infection, "chronic diarrhea is a symptom of diarrhea-predominant irritable bowel syndrome, HIV infection and HAART" and "the administration of Crofelemer was known to be effective for treating the symptoms of diarrhea... in diarrhea-predominant irritable bowel syndrome and HIV infection" (see, *e.g.*, page 4 of the Office Action mailed March 11, 2013).

6. In contrast to the Examiner's assertions, it is well known in the scientific literature that, although the exact causes of diarrhea are not always known, *Diarrheal disease due to various causes will show one or more discretely different mechanisms*. In general, there are four major types of diarrhea:

- (1) osmotic diarrhea;
- (2) secretory diarrhea;
- (3) diarrhea associated with motility abnormalities; and
- (4) exudative diarrhea.

7. Osmotic diarrhea results when poorly absorbable aqueous solutes (*e.g.*, sugars in diet drinks or milk of magnesia laxative) are ingested, resulting in osmotic force that quickly pulls water and ions into the intestinal lumen. Secretory diarrhea, on the other hand, results from overstimulation of the intestinal tract's secretory capacity and can result from infection or as a component of a more complex disease. Diarrhea associated with motility abnormalities results from

¹ The McMahan reference is solely used for teaching that SP-303 is crofelemer.

both increases and decreases in gut motility (propulsive motor activity). Finally, exudative diarrhea results when the intestinal epithelium's barrier function is compromised by loss of epithelial cells or disruption of tight junctions, leading to increasing hydrostatic pressure and accumulation of water and electrolytes in the lumen. For a review of the pathophysiology of diarrhea, see the review articles by Shah, 2004 and Field, 2003, copies of which are enclosed herein as Exhibits B and C, respectively.

8. The Quart reference relied on by the Examiner in the Office Action discloses the use of a low dose of crofelemer to treat diarrhea-predominant irritable bowel syndrome (d-IBS). The Examiner has combined the teachings of the Quart reference regarding the low dose of crofelemer for treating d-IBS with the Holodniy reference for allegedly teaching that "[c]rofelemer was known to be effective in treating the symptoms of diarrhea associated with HIV or... HAART associated diarrhea" (see, e.g., pages 4-5 of the Office Action).

9. However, in contrast to the Examiner's statements, one of ordinary skill in the art would not have thought that a low dose of crofelemer (effective for treating d-IBS as taught by Quart) would be effective to treat HIV-associated diarrhea or HAART associated diarrhea (as taught by Holodniy) because these are different types of diarrhea and have different physiological causes and likely different mechanisms of diarrhea.

10. d-IBS results in diarrhea characterized by chronic abdominal pain, discomfort, and bloating. The causes of d-IBS are not known, and there is no evidence that the *diarrhea in d-IBS is caused by secretory diarrhea, or overstimulation of the intestinal tract's secretory capacity*. As an example, Octreotide, an antisecretory drug, was shown not to favorably improve symptoms of non-constipation forms of IBS.

11. Although the exact causes of HIV-associated diarrhea are also unknown, *diarrhea in HIV patients is an exudative diarrhea resulting from impaired epithelial-barrier function by HIV-stimulated cytokine release* and, with advanced HIV infection, diarrhea is associated with malabsorption and osmotic diarrhea. (see, e.g., Stockmann *et al.*, 1997, a copy of which is enclosed herein as Exhibit D).

12. There is no evidence that the type of diarrhea seen with d-IBS and HIV-associated diarrhea and HAART associated diarrhea have anything to do with one another. The intestinal

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processes involved are *so entirely different* that it would not be obvious that crofelemer would be effective in treating HIV-associated diarrhea and HAART-associated diarrhea *at the claimed low doses* just because crofelemer is effective in treating diarrhea associated with d-IBS at a low dose.

13. In my personal studies, crofelemer is not effective in treating the routine diarrhea seen in adults, but it is effective in treating traveler's diarrhea. The mechanism of crofelemer is very selective and targets one very specific type of secretory diarrhea. Based on more than 30 years of research in this field, I would have guessed the crofelemer would have no activity in either d-IBS or HIV associated diarrhea. It is totally surprising that the drug would have activity against both very different conditions, especially at the claimed low doses.

14. There are several examples in the scientific literature that one drug is effective for one type of diarrhea but not effective for treating other types of diarrhea:

- A. Sandostatin (octreotide), a long-acting somatostatin analog, stimulates salt and water secretion and inhibits release of hormones that contribute to diarrhea. Sandostatin has been shown to be useful for treating HIV-associated diarrhea (see, *e.g.*, Fanning *et al.*, Pilot Study of Sandostatin (Octreotide) Therapy of Refractory HIV-Associated Diarrhea, *Digestive Diseases and Sciences*, 36(4):476-480, 1991, attached herein as Exhibit E). However, it has also been shown that use of Sandostatin in patients with diarrhea-predominant irritable bowel syndrome (d-IBS and non-constipated IBS) has "no visceral analgesic effect and fails to improve irritable bowel syndrome symptoms" (see, *e.g.*, Conclusions of Klooker *et al.*, Effect of long-term treatment with octreotide on rectal sensitivity in patients with non-constipated irritable bowel syndrome, *Alimentary Pharmacology & Therapeutics*, 26:605-615, 2007, attached herein as Exhibit F). Specifically, Klooker *et al.* conclude that "we were unable to show clinical improvement: global relief and symptoms of abdominal pain, bloating, flatulence, urgency or the feeling of incomplete evacuation did not improve after 8 weeks of treatment with octreotide [in IBS patients]" (see, *e.g.*, first paragraph, right column, page 613 of Klooker *et al.*).
- B. Loperamide (Imodium) is an anti-motility drug which functions by inhibiting opioid receptors in the large intestine, thereby decreasing muscle activity and motility in the

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intestine. Although loperamide has been found to be effective for treating secretory diarrhea, patients with HIV-associated diarrhea have been found to have a poor response to loperamide (see, e.g., first paragraph, right column, page 166 of Katabira, Epidemiology and management of diarrheal disease in HIV-infected patients, *Int. J. Infect. Dis.*, 3(3):164-167, 1999, attached herein as Exhibit G).

- C. Attapulgitte has been shown to be effective for treating secretory diarrhea but was found to be ineffective in treating HIV-associated diarrhea (see, e.g., Nwachukwu and Okebe, Antimotility agents for chronic diarrhea in people with HIV/AIDS, *Cochrane Database Syst. Rev.*, October 8, 2008: CD005644, attached herein as Exhibit H).

Therefore, one of skill in the art would understand that, simply because a drug is effective in treating one type of diarrhea does not mean that the same drug will be effective in treating other types of diarrhea, especially if the two different types of diarrhea result from different physiological causes.

15. In view of the foregoing, one of ordinary skill in the art would not have thought that *the claimed low dose of crofelemer* would be effective for treating *HIV-associated diarrhea or HAART associated diarrhea* based on the Quart, McMahan and Holodniy references because: (1) the mechanisms of action differ between the diseases and (2) simply because a drug is effective in treating one type of diarrhea does not mean that the same drug will be effective in treating other types of diarrhea.

Application No. 13/294,307
Inventor Declaration
Reply to Office Action of March 11, 2013

Docket No.: 119557-06005

16. All statements made herein of my own knowledge are true, all statements made herein on information and belief are believed to be true, and these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

Signed: 
Herbert L. DuPont, M.D.

Date: 15 August 13

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: _____
Electronic Signature _____

Docket No.: 119557-06005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Application No.: 13/294,307

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For: METHODS AND COMPOSITIONS FOR
TREATING HIV-ASSOCIATED DIARRHEA

Examiner: W. A. Craig

SECOND DECLARATION OF HERBERT L. DUPONT UNDER 37 C.F.R. § 1.132

I, Herbert L. DuPont, M.D., declare and state the following:

1. I am the Chief of Internal Medicine at St. Luke's Hospital, as well as the Director of the Center for Infectious Diseases at the University of Texas – Houston School of Public Health, and the Vice Chairman of the Department of Medicine at Baylor College of Medicine. I also hold the Mary W. Kelsey Chair of Medical Sciences at The University of Texas Medical School – Houston, and I have served as an epidemic intelligence service officer at the Centers for Disease Control (CDC). I received my B.A. degree from Ohio Wesleyan University and my M.D. degree from Emory University School of Medicine. I completed an internal medicine internship and residency at the University of Minnesota and an infectious disease fellowship at the University of Maryland. I am licensed to practice in Texas and Georgia. My curriculum vitae is attached as Exhibit A.

2. I am a consultant for Salix Pharmaceuticals, one of the assignees of the instant application.

3. I have no direct financial interest in the outcome of this application.

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4. I understand that the instant application is directed to methods of treating HIV associated diarrhea or highly active antiretroviral therapy (HAART) associated diarrhea in an HIV positive subject by administering a total of about 250 mg of crofelemer per day to a subject in need thereof.

5. I have studied the Holodniy (*Am. J. Gastroenterol.*, 1999), McMahan (*Alimentary Pharmacol. & Therapeutics*, 2007)¹ and Quart (U.S. Patent Publication No. 2007/0254050) references.

6. I understand that the Patent Office has alleged that these references teach that chloride ion inhibition is the mechanism by which crofelemer is alleviating the symptoms of diarrhea associated with HIV, HAART and d-IBS.

7. It is well documented in the scientific literature that for most cases of diarrhea the mechanism of action of diarrhea is not known.

8. There is no evidence and it is highly unlikely that the type of diarrhea associated with d-IBS and the type of diarrhea associated with HIV and HAART are the result of common mechanisms of action.

9. Relevant to this claim is the importance and nature of multiple chloride channels in health and diarrheal disease.

10. Chloride secretion is necessary for normal intestinal function. A variety of calcium-activated chloride channels are involved in the secretion of chloride into the intestinal lumen, creating the driving gradient for fluid movement.

11. It is well documented in the scientific literature that calcium-activated chloride channels also play a role in diarrheal disease.

12. For example, rotavirus infection only moderately increases luminal chloride concentrations and does not involve Cystic fibrosis transmembrane conductance regulator (CFTR) channels (see, *e.g.*, Morris *et al.*, 1999, enclosed herein as Exhibit B).

13. However, noroviruses, the most common cause of acute diarrhea and gastroenteritis, inhibit secretory pathways and reduce movement of anions across the gut mucosa (see, *e.g.*, Sharp *et al.*, 2010, enclosed herein as Exhibit C).

¹ The McMahan reference is solely used for teaching that SP-303 is crofelemer.

Other pathogens effect chloride secretion indirectly through inhibition of agonist-induced secretory responses (see, *e.g.*, Hecht and Koutsouris, 1999, enclosed herein as Exhibit D), while some pathogenic microbes decrease the intestinal release of chloride to cause diarrhea (see, *e.g.*, Tomson *et al.*, 2005, enclosed herein as Exhibit E).

14. Although chloride secretion plays a role in many forms of diarrhea, it is not the only mechanism of action that leads to diarrhea. In the forms of diarrhea where chloride is involved, chloride is released via channels other than the CFTR pathway affected by crofelemer.

15. For example, the Holodniy *et al.* reference states that "[w]hether the stool chloride findings in our study reflect a physiological effect of treatment with SP-303 is unknown" (see, *e.g.*, page 3271, right column, second full paragraph, last sentence of Holodniy).

16. The Holodniy *et al.* reference also states that "the precise role of chloride ion secretion in the watery diarrhea syndrome of AIDS-associated diarrhea is not known" (see, *e.g.*, page 3272, left column, first paragraph of Holodniy).

17. Thus, the Holodniy *et al.* reference acknowledges the lack of evidence for drawing a connection between the change in chloride secretion and an effect on HIV and HAART associated diarrhea.

18. There is no evidence in Holodniy that chloride ion inhibition was the mechanism by which crofelemer alleviated symptoms of diarrhea in HIV-associated diarrhea and HAART associated diarrhea.

19. If the inhibition of chloride channels were the sole mechanism by which calcium activated chloride channel inhibitors act to improve symptoms of diarrhea in HIV and HAART patients and other forms of diarrhea, then calcium activated chloride channel inhibitors would be effective for the treatment of all types of diarrhea, which is not the case.

20. In my extensive research experience, calcium activated chloride channel inhibitors, such as crofelemer, are not effective in treating acute non-specific diarrhea, the most common type of diarrhea seen in adults in all regions of the world.

21. This form of common diarrhea is caused by toxins and viruses, but does respond to antimotility drugs like loperamide, showing the diverse nature of diarrhea and its response to different treatments.

22. Although not completely understood, the mechanism of crofelemer is highly selective and is not effective for treating all types of diarrhea, and crofelemer is, in fact, not effective for treating the most common forms of acute diarrhea.

23. Based on more than 40 years of research in this field, I would have expected that crofelemer, at the low dosage of about 250 mg per day, would have no activity in HIV associated diarrhea or HAART associated diarrhea based on the Holodniy, McMahan and Quart references.

24. It is surprising to me that the drug would have activity against these very different conditions with strikingly different disease mechanisms and clinical manifestations, especially at the claimed low dose. In my opinion this could never have been anticipated based on the completely different disease pathogeneses.

25. All statements made herein of my own knowledge are true, all statements made herein on information and belief are believed to be true, and these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

Signed: 

Herbert L. DuPont, M.D.

Date: 3 June 14

6768

May 2014

CURRICULUM VITAE

Name: Herbert L. DuPont, MD, MACP

Address: University of Texas School of Public Health
1200 Pressler Street, Suite 733
Houston, Texas 77030
Phone 713-500-9366
Fax 713-500-9359

Education:
A.B., Ohio Wesleyan University, 1961
M.D., Emory University School of Medicine, 1965

Postgraduate Education:
Medical Internship - University of Minnesota Hospitals, 1965-1966
Medical Residency - University of Minnesota Hospitals, 1966-1967
Fellowship, Infectious Diseases - University of Maryland School of Medicine,
1967-1969

Medical License: Georgia and Texas

Board Qualifications:
American Board of Internal Medicine Qualifying Examination, October 1970
Diplomat in Internal Medicine Oral Examination, February 1972

Hospital Admitting Privileges:
Internal Medicine and Infectious Diseases, Baylor St. Luke's Medical Center

Service with United States Public Health Service:
Epidemic Intelligence Service Officer (Centers for Disease Control)
Assigned to the University of Maryland where studies of induced enteric infectious
diseases in volunteers were conducted for the purpose of vaccine development and
studies of pathogenesis, 1967-1969

Current Professional and Academic Appointments:
*Director, Center for Infectious Diseases, Professor of Infectious Diseases, The
University of Texas-Houston School of Public Health, 2000-present*

*Chief, Internal Medicine Service, Baylor St. Luke's Medical Center, Houston,
Texas, January 1995-present*

*Mary W. Kelsey Distinguished Chair in the Medical Sciences, The University of
Texas-Houston Medical School, April 1988-present*

*The Baylor College of Medicine H. Irving Schweppe, Jr., M.D. Chair in Internal
Medicine and Vice Chairman, Department of Medicine, April 1995-present*

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Clinical Professor, Department of Medicine and Adjunct Professor, Department of Microbiology and Immunology, Baylor College of Medicine, 1977-present

Professor, Graduate Schools of Biomedical Sciences (GSBS), University of Texas – Houston (Molecular Pathology), 2002 – present, and Professor, GSBS, Baylor College of Medicine (Department of Molecular Virology and Microbiology, 2004 – present

Adjunct Professor, Department of Infectious Diseases, Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, 2008-present

President and CEO, Kelsey Research Foundation, 2008-present

Adjunct Professor, Department of Clinical Services and Administration, University of Houston, College of Pharmacy, 2008-present

Past Medical School Faculty Appointments:

Instructor in Medicine, Division of Infectious Diseases, University of Maryland School of Medicine, July 1969

Assistant Professor of Medicine, Division of Infectious Diseases, University of Maryland School of Medicine, July 1970

Associate Professor of Medicine with tenure, Division of Infectious Diseases, University of Maryland School of Medicine, July 1972

Professor of Medicine, Founding Director, Program in Infectious Diseases and Clinical Microbiology, The University of Texas Medical School at Houston, 1973-1988

Acting Chairman, Department of Internal Medicine, The University of Texas Medical School at Houston, December 1977-August 1978

Interim Chairman, Department of Internal Medicine, The University of Texas Medical School at Houston, September 1987-June 1989

Director, Center for Infectious Diseases, The University of Texas Medical School at Houston, and The University of Texas School of Public Health, 1989-1994

Vice Chairman, Department of Internal Medicine, The University of Texas Medical School at Houston, 1990-January 1995

University of Texas School of Public Health Appointments

Professor and Director, Center for Infectious Diseases

Professor of Epidemiology and Infectious Diseases, Division of Epidemiology, Human Genetics and Environmental Sciences

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Member, Executive Council

President's Award Selection Committee, UTHSCH, 2009-present

St. Luke's Episcopal Hospital Appointments:

Medical Executive Committee (MEC) 1995-present

MacDonald General Research Fund, Chair, 1995-present

Greater Houston Health Care Network (Community Health Centers in Houston):
Board of Directors, 1997-2002; Chairman 2000-2003; Secretary 2008-2010;
President 2011-present

Member, Joint Conference Committee (Medical Staff leaders/Hospital Board)
1996-1997, 2000-present

Record Screening (QA) Committee, Chair, Internal Medicine Service, 1995-2000

Risk Management Committee, 1995-present

St. Luke's Home Health Service, Advisory Board (Medical Advisor) 1995-2003

Ethics Committee, Chair, 1998-2002

Quality of Care Committee, 1998-2007

Editorial Board, Hospital Rounds Publication, 1996-present

Infection Control Committee Chair, 1996-present

Committee on Practitioner Health, Vice Chair, 2000-present

St. Luke's Travel Medicine, Medical Director, 1999-2005

Co-Chair, Committee for Weapons of Mass Destruction Preparedness, 2001-2006

Baylor College of Medicine,

Department of Medicine Appointments:

Vice Chairman, 1995-present

Clinical Chiefs Subcommittee, 1995-2007

Executive Committee, 1995-present

Clinical Faculty Promotions Committee, 1996-2007

Resident Selection Committee, 1997-present

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Professional Conduct Committee, 2000-2007

**University of Texas and
Hermann Hospital Appointments:**

Chairman, Clinical Policy Committee, 1987-1989

Chairman, Committee on Acquired Immune Deficiency Syndrome (AIDS) 1988-1994

Joint Institutional Planning Committee, 1987-1989

Interim Chief of the Hospital Service of Internal Medicine, September 1987-June 1989

Chairman, Infection Control Committee, 1973-1986

Credentials Committee, 1973-1974

International Medical Advisory Board, 1994-1995

Curriculum Committee, Chairman, 1974-1975

Faculty Promotions and Tenure Committee, 1973-1974 and 1980-1981

General Research Support Committee, 1973-1975

Board of Trustees, Medical Service Research and Development Plan, 1975-1978

Faculty Senate Vice-Chairman, 1975-1977; Senator, 1975-1980

Administrative Council, 1975-1989; Secretary 1976-1977, 1979-1981

Health Science Center, Infectious Diseases Review Panel, 1992-1994

Continuing Medical Education Committee, 1993-1994

Student Evaluations and Promotions Committee, 1994-1995

Faculty Development Leave Committee, 1993-1996, Chairman 1994-1996

Research Conflict of Interest Committee, University of Texas Health Science Center, 2002-2005

International Endeavors Task Force, 2002-2003

International Relations Council, University of Texas Health Science Center at Houston, 2004-2008

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Executive Council, University of Texas School of Public Health, 2007-present

President's Scholar Awards Committee, University of Texas Health Sciences Center at Houston, 2009-present

Consultantships and Committees

Medical Knowledge Self Assessment Program (MKSAP) IV Committee on Infectious Diseases, American College of Physicians, 1975-1976

Member and Chairman, Subcommittee on Bacterial Diseases, U.S. Army Medical Research and Development Command, 1977-1981

Detection and Defense, Infectious Agents Used in Warfare, U.S. Army Research and Development Command, June 23-24, 1981

Chairman, External Review Committee, Research Program on Legionella Organisms and the Diseases They Produce. U.S. Army Research Institute of Infectious Diseases, September 9-11, 1981

Medical Research Service Merit Review Board for Infectious Diseases, Veterans Administration, 1982-1985

Program Committee, Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) 1983-1988

Advisory Committee, Development of Rotavirus Vaccine, Merck Sharp & Dohme Research Laboratories and National Institutes of Health, February 28-29, 1984

Advisor, Ministry of Health, Saudi Arabia concerning Communicable Diseases of the Pilgrims during the 1984 Islamic Hajj, August 25-September 10, 1984

Planning Committee for Consensus Development Conference on Travelers' Diarrhea, National Institutes of Health, May 21-22, 1984 and January 27-30, 1985

Ad Hoc Committee on Environmental Impact, Live Recombinant DNA Enteric Vaccines, U.S. Army Research and Development Command, December 16-17, 1986

Blue Ribbon Committee on the Use of AZT for Patients with Acquired Immune Deficiency Syndrome. Infectious Diseases Society of America, and Burroughs Wellcome Company, December 1986-January 1988

Clinical Evaluation of Anti-Infective Drugs, Chairman Infectious Diarrhea Subcommittee, Infectious Diseases Society of America/Food and Drug Administration 1989-1990

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Middle-East Regional Infectious Disease Research Program, Scientific Advisory Committee, Institute of Medicine, National Academy of Sciences, November 1, 1989 to September 30, 1994

Vaccines and Related Biological Products Advisory Committee of the U.S. Food and Drug Administration, February 1, 1989-January 31, 1993; FDA Special Government Employee (Consultant) 1993-present

Board of Scientific Counselors, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC) 1992-1996

Member, Standing Scientific Advisory Committee of The Thrasher Research Fund, 1993-December 1996

U.S.-Mexico Foundation for Science, and Technology, Committee Chair (U.S.)-Health, 1994-1999

Medical Advisory Steering Committee of the City of Houston Medical Strike Team For Biological, Chemical and Nuclear Terrorism, 2001-2006

Board of Advisors, Emory University School of Medicine, 2001-2010
Member, Emory Medical School Campaign Committee, 2006-present

NIAID Blue Ribbon Panel on Bioterrorism and its Implications For Biomedical Research, Washington, DC, October 2002

Clinical Leaders Member, Clinicians' Biodefense Network, Center for Civilian Biodefense Strategies, Johns Hopkins University Bloomberg School of Public Health, 2002-2007

Pandemic Influenza Committee, Texas Medical Center, Houston, 2006-present

Advisory Committee on Animal and Human Health of the Houston Zoo, 2006-2007

Board of Scientific and Policy Advisors, American Council on Science and Health (ACSH), 2006-present

Board of Advisors, The John P. McGovern Museum of Health & Medical Sciences, Houston, 2007-present

Member Steering Committee, World Gastroenterology Organization, 2010-2011

Service on Editorial Boards:

Associate Editor, American Journal of Epidemiology, July 1978-July 1981

Associate Editor, Journal of Infectious Diseases, 1983-1988

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Editorial Board, Clinical Infectious Diseases (formerly The Reviews of Infectious Diseases) 1990-1995

Editorial Board, Infectious Diseases in Clinical Practice, 1992-present

International Advisor, The Journal of Infection, 1997-2002; Editorial Board, Journal of Infection, 2002-present

Editorial Board, Infectious Disease News, 1995-present

Editorial Board, Rounds, St. Luke's Episcopal Hospital, 1999-present

Deputy Editor-in-Chief, Journal of Travel Medicine, 2003-2011

Editorial Advisory Board, Journal of Infectious Diseases, 2006-present

Editorial Advisory Board of Gastroenterology & Hepatology, 2007-present

Leadership in Professional Organizations:

Houston Society of Internal Medicine, Vice President and Program Chairman 1978-1979, President 1985-1986

Councilor, Infectious Diseases Society of America, 1978-1981

Secretary, Infectious Diseases Society of America, 1982-1987

President-Elect, Infectious Diseases Society of America, 1988-1989

President, Infectious Diseases Society of America, 1989-1990

Texas Academy Chapter, American College of Physicians, Vice President 1988-1989, President-elect 1989-1990, President 1990-1991

Founding President, International Society of Travel Medicine, 1991-1993

International Board of Directors, Amigos de las Americas, 1984-2002

Board of Directors, The National Foundation for Infectious Diseases, 1981-2002, Vice President The National Foundation for Infectious Diseases, 1994-1997, President, The National Foundation for Infectious Diseases, 1997-1999

Board of Trustees, Kelsey Research Foundation, 2000-present

American Clinical and Climatological Association, Recorder 2000-2005, President-Elect 2005-2006, President 2006-2007, member of the Council, 2000-2011

Board of Directors, Texas Academy of Internal Medicine, The Texas Chapter of The American College of Physicians, 2001-2007

Governor, Texas Southern Region, Board of Governors, American College of Physicians, 2003-2007

Named Lectureships and Special Presentations

36th Annual Stoneburner Lecturer, Medical College of Virginia, February 25, 1983

Hurst Brown Visiting Professor, University of Toronto and Toronto Western Hospital, April 1-4, 1984

Harry Dowling Visiting Professor of Infectious Diseases, University of Illinois at Chicago, April 17-18, 1986

Federico Gomez Lecture, Hospital Infantil de Mexico, Mexico City, November 17, 1988

C. Richard Bowman Memorial Lecture, University of Virginia School of Medicine, October 11, 1990

President's Address, 28th Annual Meeting of the Infectious Diseases Society of American (IDSA), Atlanta, GA, October 25, 1990.

Commencement Address, University of Texas Medical School - Houston, June 7, 1996

William M.M. Kirby Visiting Professor, University of Washington School of Medicine, December 11-13, 1996

Jesse D. Ibarra, Jr., M.D. Lectureship in International Health, Scott and White Clinic (Texas A&M College of Medicine) November 6, 1997

Keynote Speaker, White Coat Ceremony for entering Medical Class of 1998, University of Texas Medical School - Houston, August 12, 1998

Pfizer Visiting Professor, Morehouse School of Medicine, Atlanta, Georgia, May 23-25, 2000

J.V. Irons Lecture, 52nd Southwest Conference on Diseases in Nature Transmissible to Man, Houston, June 6, 2002

William P. Deiss, Jr., M.D. Lecture, The University of Texas Medical Branch, Galveston, TX, April 26, 2003

Wesley W. Spink, M.D. Memorial Lecture, University of Minnesota Medical Center, April 21, 2005

26th Annual Alpha Omega Alpha (AOA) - Theodore E. Woodward Lecture, University of Maryland School of Medicine, Baltimore, MD, April 26, 2006

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15th Annual Walter M. Kirkendall, MD Endowed Lecture, The University of Texas – Houston Medical School, Houston, March 27, 2007

15th Annual James H. Steele, DVM Lecture, University of Texas – Houston School of Public Health, Houston, April 4, 2007

President's address, 120th Annual Meeting of the American Clinical and Climatological Association, Tucson, AZ, October 19, 2007

Society Supporting Leadership in Internal Medicine (SSLIM) Annual Address, The University of Utah School of Medicine, St. Lake City, September 5, 2008

Sigma Xi Distinguished Lecture, Texas A & M University-Corpus Christi, October 10, 2008

11th Annual Arthur C. White, MD Lecture, Indiana University School of Medicine, Indianapolis, November 20, 2009

2nd Annual Ellen Dickinson, M.D. Memorial Lecture, Research Day, Ohio Wesleyan University, September 20, 2010

Ben H. Kean - Boxer Family Foundation Lecture in Global Health and Ben J. Kean Medal, Weill Cornell Medical College, New York City, October 9, 2012

Societies and Honors:

Alpha Omega Alpha (University of Texas, 1975)
American Clinical and Climatological Association
American Epidemiological Society
American Federation for Clinical Research
American Medical Association
American Men and Women of Science
American Society for Clinical Investigation
American Society for Microbiology
Association of American Physicians
Harry A. Feldman Presidential Award, Infectious Diseases Society of America
Fellowship, American College of Physicians
Fellowship, American Academy of Microbiology
Harris County Medical Society
Infectious Diseases Society of America
International Society for Infectious Diseases
International Society of Travel Medicine
Southern Society for Clinical Investigation
Texas Club of Internists
Texas Medical Association
Who's Who in America
Who's Who in the World

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Centennial Classic Article, Reference 11, published in 1971 (original articles) first describing the biologic properties of Norwalk virus was reprinted in the Journal of Infectious Diseases in 2004 (reference 237, in Reviews, page 45)

Science Citation Classic, Reference 12, published in 1971 (original articles) dealing with the pathogenesis of *Escherichia coli* diarrhea, was cited in 1985 ISI Current Contents as one of the 100 most cited articles published in The New England Journal of Medicine

Formal recognition by The University of Texas Medical Students for Teaching Excellence; 1975, 1980, 1981, 1982, 1983, 1984, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1997, 2007, 2010

The John P. McGovern Most Distinguished Clinical Teacher, University of Texas Medical School, Houston, June 1991

Included in The Best Doctors in America 1992-2014 (for Infectious Disease, Tropical and Travel Medicine, Diarrhea),

Included in America's Top Doctors The Nation's Leading Medical Specialists, Castle Connolly Medical Ltd, 2001-2013 (for Infectious Disease)

Bronze Medaille D'Honneur de la Ville de Puteaux (Bronze Medal of Honor, Paris, France) April 25, 1993 for leadership in travel medicine

Medal of Honor, from the International Society of Travel Medicine in gratitude for leadership as first society president and for work in the development of the Journal of Travel Medicine, 1995.

Benjy F. Brooks, M.D. Award, The Outstanding Clinical Faculty Member, Alumni Association, The University of Texas-Houston Medical School, June 5, 1997

Honorary Doctorate, in recognition of achievements in areas of understanding how *Escherichia coli* produces diarrhea and furthering our knowledge of the causes and pathophysiology of intestinal infections: University of Zurich, Zurich, Switzerland, April 24, 2004

Citation from Texas Academy of Internal Medicine (Texas Chapter, American College of Physicians), for advancement of clinical knowledge, improved patients' health, outstanding leadership and initiative, June 4, 2005

Distinguished Achievement Citation, the university's highest honor for alumni, for: contributions to scientific research, teaching, clinical practice and service to the global community, Ohio Wesleyan University, Delaware, Ohio, May 20, 2006

The Maxwell Finland Award for Scientific Achievement for outstanding contributions to understanding of infectious diseases and public health, National Foundation for Infectious Diseases, Washington, DC, March 21, 2007

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Mastership in the American College of Physicians (ACP), awarded at a formal Convocation of the ACP, May 15, 2008, Washington, DC

Laureate Award from the Texas Academy of Internal Medicine, (ACP) for commitment to excellence in medical care, education and research and for service to the community and the ACP, Dallas, Texas, November 15, 2008

2008 President's Scholar Award for Research, University of Texas Health Science Center at Houston, for sustained contributions to pathogenesis and treatment of diarrheal diseases coupled with internationally recognized leadership in infectious diseases and translational medicine, May 20, 2009

2009 Distinguished Medical Achievement Award, Emory University School of Medicine, Atlanta, Georgia, September 25, 2009

2009 TIAA-CREF Distinguished Medical Educator Award, Houston, December 3, 2009 (Texas state-wide medical teaching award)

Alexander Fleming Award for Lifetime Achievement in Infectious Diseases, Infectious Diseases Society of America, October 21, 2010, Vancouver, BC

Award for Innovation, for work with *Clostridium difficile* infection, St. Luke's Episcopal Hospital, December 3, 2012, Houston, Texas

Trainees: (details available upon request)

University of Texas – Houston Medical School

M.D. Postdoctoral Fellows (18)

1. Charles D. Ericsson, M.D. (1978; University of Texas - Houston Medical School)
2. Benjamin L. Portnoy, M.D. (1979, Memorial Hermann Hospital)
3. Ricardo Bolivar, M.D. (1979, Private Practice ID in Houston)
4. Guillermo Ruiz-Palacios, M.D., (1979, Instituto Nacional de la Nutricion, Mexico City)
5. John M. Boyce, MD, (1979, Yale University School of Medicine, Hospital of Saint Raphael, New Haven, CT)
6. Francisco Javier de la Cabada, M.D. (1983, University of Guadalajara, Guadalajara, Jalisco, Mexico)
7. Barbara Mederski-Samoraj, M.D. (1982, University of Toronto School of Medicine)
8. Philip C. Johnson, M.D. (1987, University of Texas – Houston Medical School)
9. Roberto C. Arduino, M.D. (1987; University of Texas – Houston Medical School)
10. Pablo C. Okhuysen, M.D., (1994, University of Texas - Houston Medical School)
11. Ernesto G. Scerpella, M.D. (Vaccine 1995, Pfizer Pharmaceutical Company, Miami, FL)
12. Made Sutjita, M.D., Ph.D. (2000, Loma Linda University, Riverside County Regional Medical Center)
13. Harumi Gomi, M.D., MPH. (2001, Jichi Medical School, Kawachi, Tochigi, Japan)
14. Javier A. Adachi, M.D. (2000, MD Anderson Cancer Center).

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15. Rosa Infante, M.D. (2002, Cayetano Heredia Medical School, Lima, Peru)
16. Karen Vigil, MD (2009 – MD Anderson Cancer Center)
17. Nicholas Melgarejo, MD (2011 – Private Practice, San Antonio, Texas)
18. Javier de la Cabada Bauche, MD (2011, University of Guadalajara, Guadalajara, Jalisco, Mexico)

Ph.D. Postdoctoral Fellows (6)

1. John J. Mathewson, Ph.D. (1985, Oklahoma State Department of Health)
2. K-H Kim, Ph.D. (1984, at a medical school in Korea)
3. Lindsey V. Wood, Ph.D. (1985, Community Research, Cincinnati, OH)
4. Donna R. Morgan Murray, Ph.D. (1986, Bristol-Myers Squibb)
5. Teresa Estrada-Garcia, Ph.D. (2000, CINVESTAV-IPN, Mexico City, Mexico)
6. Ismail M. Meraz, PhD (2008, MD Anderson Cancer Center)

University of Texas Graduate School of Biomedical Sciences

Ph.D. Student (1)

1. Charles Darkoh, Ph.D. (2008-2012 –University of Texas Graduate School of Biomedical Sciences student)

Baylor College of Medicine

M.D. Postdoctoral Fellows (10)

1. Juan Pablo Caeiro, M.D. (1999, Clinical Faculty, Baylor College of Medicine, Houston, TX)
2. Alain R. Bouckennooghe, M.D., MPH. (2002 Sanofi Pasteur, Swiftwater, PA)
3. David E. Greenberg, M.D. (2002, Assistant Professor Infectious Diseases, University of Texas Southwestern Medical School)
4. Mohamad Miqdady, M.D. (2002, Abu Dhabi, United Arab Emirates)
5. Natalie Sikka, M.D. (2005, University of Rochester Medical School, Rochester, NY)
6. David B. Huang, M.D. (2005, Pfizer Global Pharmaceuticals, Collegeville, PA)
7. Coralith Garcia, M.D., (2006, Cayetano Heredia Hospital and Medical School, Lima, Peru)
8. Hoonmo Koo, MD (2008, Assistant Professor, Baylor College of Medicine, Houston, Texas)
9. Jeannette Ouyang, MD (2010, Private Practice, Dallas, Texas)
10. Jonathan Crews, MD (2014, Assistant Professor of Pediatrics, Baylor College of Medicine)

Ph.D. Students (1)

1. Nadim Ajami, Ph.D. (2006-2011 – Graduate School of Biomedical Sciences)

University of Texas School of Public Health

Ph.D./Dr.PH. Students (12)

1. Weillie S. Tjoa, M.D., Ph.D. (1976, Private Practice Houston, Texas)
2. Ekanem E. Ekanem, Ph.D. (1984, Nigeria in an academic appointment)
3. George F. Lemp, Dr.P.H. (1985, University of California, Oakland, CA)
4. Peggy S. Sullivan, Ph.D. (1986, consultant to the World Health Organization, deceased.
5. Charles E. Wright, Ph.D. (1991, Houston Veterans Administration Hospital)
6. Moshaddeque M. Hossain, MBBS, Ph.D. (1994, University Brunei Darussalam, Brunei Darussalam)
7. Mohammad Alamgir Mahmud, Ph.D, MBBS (1996, St. Jude Heritage Occupational and

Environmental Health Services, La Mirada, CA)

8. Alireza J. Mosavi, Ph.D (1998, Faculty position in Lebanon)
9. David B. Huang, M.D., Ph.D., MPH (2005, Pfizer Global Pharmaceuticals, Collegeville, PA)
10. Sumesh Kachroo, (2008, United Biosource Corporation, Bethesda, MD)
11. Kirsten Raines, PhD (2010, University of Texas Health Science Center at Houston)
12. Vineetkumar B. Kharat (2013, University of Texas Health Science Center at Houston)

MPH Students (24)

1. JoAnn A. Bitsura, MPH (1986, US pharmaceutical company)
2. Gerry A. Haynes, R.N., MPH (1987, deceased)
3. Raouf R. Arafat, M.D., MPH (1993, Houston City Health Department)
4. Fida M. Khan, M.D., MPH (1995, Kingdom of Saudi Arabia)
5. M F Hussain, M.D., MPH (1995, Physician in Pakistan)
6. Liliana Rodriguez, MPH, (YR, University of Texas Health Science Center at Houston)
7. Von Nguyen, MPH. (2002, Medical Student, University of Texas Southwestern Medical School)
8. Alain R. Bouckennooghe, M.D., MPH. (2001, Sanofi Pasteur, Singapore)
9. David B. Huang, M.D., PhD, MPH (2005, Pfizer Global Pharmaceuticals, Collegeville, PA)
10. Cesar de las Casas, M.D., MPH (Epidemiology 2003, The University of Miami Medical School, Miami, Florida)
11. Jill Amaon, MPH, (2004, uncertain location)
12. Ernest Wong, MPH, (2005, medical student)
13. Shruti P. Agnihotri, MBBS, MPH, (2007, neurology resident in Chicago)
14. Sridivya Jaini, MBBS, MPH, (2007, internal medicine resident at the University of Connecticut)
15. Don Thushara Galbadage, MPH, (2009 MD-PhD program at Texas A & M University, College Station, Texas)
16. Nipam Shah, MBBS, MPH (2009, academic physician, Delhi, India)
17. Yashoo Yadav, MBBS, MPH, (2009, internal medicine resident, University of Connecticut)
18. Zahra Asgari, MPH (2010)
19. Stephen Harold, MBBS, MPH (2010, University of Texas Health Science Center at Houston)
20. Eric Chen, MS (2012 – Toledo University School of Medicine)
21. Jamie Huang, MS (2010 - University of Texas Health Science Center at Houston)
22. Vijairam Selvaraj, MBBS, MPH (2012 University of Texas Health Science Center at Houston)
23. Oluwatosin Bamidele, MBBS, MPH (2014, University of Texas School of Public Health)
24. Jonathan Crews, MD, MPH (2014 Assistant Professor of Pediatrics, Baylor College of Medicine)

PhD Other Institution (Penn State University) (1)

1. Kurt Z. Long, Ph.D. (1993, School of Population Health, University of Queensland, Brisbane, Australia)

Research Grants and Contracts:

(Where Dr. DuPont is/was Principal Investigator)

Evaluation of Control Measures Against Diseases Other Than AIDS: Enteric Challenge Studies, Vaccine Treatment and Evaluation Unit (VTEU), NIH; \$1,923,691; 6/02-6/014 (NO1-AI-25465).

A Phase II Double Bind Randomized, Placebo-Controlled Study to Evaluate the Epidemiology of Natural Infection with Enterotoxigenic *Escherichia coli* Occurring after transcutaneous Immunization in the Field Setting, Intercell Corporation, \$1,513,720, 6/1/06 to 3/23/12.

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Various donors, clinical trials with antidiarrheal and antimicrobial agents in acute diarrhea (recent companies Santarus Pharmaceuticals, Salix Pharmaceuticals, Dr. Falk Pharma), ~\$500,000/year.

Virulence factors in Cryptosporidium and infective dose in humans; EPA Grant; \$550,323; 10/1/95-5/1/97 (R 824759).

HIV-Associated Diarrhea and Wasting in Zambia; NIH; \$820,875; 3/1/92-2/28/97 (RO1 AI 31356).

Determination of Infectious Dose of Cryptosporidium parvum for Immunocompetent Humans and to Develop a Risk Assessment for Oocysts in Drinking Water. EPA Cooperative Agreement; \$661,509; 10/1/92-9/30/96 (CR 819894-01-0).

HIV-Associated Diarrhea and Wasting in Zambia; Shannon Award, NIH; \$100,000; 10/1/91-3/1/92 (RO1 AI 31356-01A1).

Immunogenicity and efficacy of a heat-killed whole cell/B subunit Cholera vaccine, U.S. Department of Defense; \$88,800 4/17/92-2/16/93 (DAMD 17-92-C-2078).

Diarrheal Disease Project in Rural Egypt; CDC/AID/NIH; \$350,000; 7/1/87-5/15/91 (Project 03-340-C).

Comparative Susceptibility of United States and Mexican Students to Enteric Pathogens; NIH; \$693,042; 5/1/86-11/30/89 (RO1 AI-23049).

A Study of Illness and Reservoirs Associated with *Giardia lamblia* in Rural Egypt; Thrasher Research Fund; \$49,620; 6/1/85-4/30/87 (Award 2795-5).

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PRACTITIONERS SECTION

EVALUATION OF DIARRHEA: THE CHALLENGE CONTINUES! PART-I

SUNDEEP SHAH

Diarrhea and constipation are among the most common patient complaint faced by primary care physicians; and they account for major referrals to gastroenterologists. Although they can be nuisance symptoms, they can signal a serious underlying condition like colorectal cancer, or systemic disorder, like thyroid disease. Most individuals experience diarrhea sometime in their life, with an episode of diarrhea usually lasting for a day or two and subsiding rapidly on its own. However, diarrhea can be a symptom of many other underlying conditions and can be chronic in nature; thus arriving at a proper diagnosis is very important for effective management of diarrhea. Normal Physiology The small intestine and colon perform functions like the secretion and absorption of water and electrolytes, storage and subsequent transport of intraluminal contents aborally, and the salvage of some nutrients after bacterial metabolism of carbohydrate that are not absorbed in the small intestine. Alterations in fluid and electrolyte handling contribute significantly to diarrhea. Alterations in motor and sensory functions of the human colon result in highly prevalent syndromes such as irritable bowel

syndrome, chronic diarrhea, and chronic constipation. Normally, the small intestine and colon absorb 98% of the total fluid load of about 10 L/day presented to it. Approximately, 1 L of residual fluid reaches the colon; the stool excretion of fluid constitutes about 0.2 L/d. The colon has a large capacitance and functional reserve and may recover up to four times its usual volume of 0.3 L/d, provided the rate of flow permits reabsorption to occur. Thus, the colon can partially compensate for intestinal absorptive or secretory disorders. A reduction of water absorption by as little as 1% can result in diarrhea. The distal ileum acts as a reservoir, emptying intermittently by bolus movements. This action allows time for salvage of fluids, electrolytes, and nutrients. Segmentation by haustra compartmentalizes the colon and facilitates mixing, retention of residue, and formation of solid stools. The ascending and transverse colon function as reservoirs while the descending colon acts as a conduit. The colon is efficient at conserving sodium and water, a function that is particularly important in sodium-depleted patients in whom the small intestine alone is unable to maintain sodium balance. Diarrhea or constipation may result from alteration in the reservoir function of the proximal colon, or the propulsive function of the left colon.

DEFINITION OF DIARRHEA

The word diarrhea originates from the Greek

terms *dia* (through) and *rhein* (to flow). Diarrhea is considered by most when there is increased liquidity of stool with or without increased frequency of stool. Stool consistency is somewhat an objective parameter; hence, stool frequency or stool weight is used to define diarrhea. Three or more bowel movements per day or stool weight greater than 200 g per day are considered as diarrhea. There may be some exceptions to this definition. For e.g. Indian diet has a high fiber content, and hence they have increased stool weight (> 200 g/day) but do not have diarrhea because they have normal stool consistency and frequency (< 3/day). Conversely, other patients have normal stool weights but complain of diarrhea because their stools are loose or watery (but frequency < 3/day). Two common conditions, usually associated with the passage of stool totaling < 200 g/d, must be distinguished from true diarrhea. Pseudodiarrhea, or the frequent passage of small volumes of stool with rectal urgency and accompanies the irritable bowel syndrome or anorectal disorders like proctitis. Fecal incontinence is the involuntary discharge of rectal contents and is most often caused by neuromuscular disorders or structural anorectal problems. Diarrhea and urgency, especially if severe, may aggravate or cause incontinence. A careful history and physical examination generally allow these conditions to be discriminated from true diarrhea.

PATHOPHYSIOLOGY AND MECHANISMS OF DIARRHEA

Normally, absorption is quantitatively greater than secretion in the intestines. Therefore, either a decrease in absorption or an increase

in secretion can lead to diarrhea. When infectious agents, toxins, or other noxious substances are present within the gut, fluid secretion and motility are stimulated to expel the unwanted material, producing diarrhea. There are four major mechanisms of diarrhea: (1) the presence in the gut lumen of unusual amounts of poorly absorbable, osmotically active solutes (osmotic diarrhea); (2) intestinal ion secretion or inhibition of normal active ion absorption (secretory diarrhea); (3) deranged intestinal motility; and (4) exudation of mucus, blood, and protein from sites of inflammation.

OSMOTIC DIARRHEA

Ingestion of poorly absorbed sugars or alcohols (e.g., mannitol, sorbitol) or ions (as found in laxatives - magnesium, sulfate, and phosphate) leads to diarrhea of the osmotic type. Since the intestines cannot maintain an osmotic gradient, these unabsorbed ions in the intestinal lumen cause retention of water to maintain an intraluminal osmolality equal to that of body fluids (about 290 mOsm/kg). Disaccharide (sucrose and lactose) requires disaccharidase for their breakdown before absorption. Lactose intolerance, results due to deficiency of disaccharidase lactase, a common clinical situation associated with diseases of the upper small intestine. Lactulose, a synthetic disaccharide cannot be hydrolyzed by the human intestine and cannot be absorbed intact in greater than trace amounts. It thereby causes an osmotic diarrhea when given in sufficient quantity to overwhelm the metabolic capacity of colonic bacteria (about 80 g/day). The essential characteristic of osmotic diarrhea is that it disappears with fasting or cessation of

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ingestion of the offending substance. In contrast, secretory diarrhea typically continues with fasting (though may be slightly reduced).

SECRETORY DIARRHEA

In this form, either net secretion of chloride or bicarbonate or inhibition of net sodium absorption is the mechanism for diarrhea. The most common cause for secretory diarrhea is infection. Infectious agents (bacteria, parasites and viruses) produce enterotoxins that interact with receptors and lead to increased anion secretion or these enterotoxins may block specific absorptive pathways (e.g. Na-H exchange). Peptides produced by endocrine tumors such as vasoactive intestinal peptide or calcitonin cause secretory diarrhea by stimulating secretion by epithelial cells. Even though there is a large reserve absorptive capacity in both the small intestine and the colon, significant loss of surface area of intestines (e.g. after resective surgery, inflammatory bowel disease), may compromise water absorption and cause diarrhea. In some cases the problem is temporary, because over time the intestine may improve its capacity for absorption by the process of adaptation.

Deranged Motility Because rapid transit prevents adequate time for absorption, diarrhea results despite intact mucosal absorptive capacity. In disorders such as diabetes mellitus, postvagotomy diarrhea, postprandial diarrhea and irritable bowel syndrome, intestinal hurry has been linked to abnormal enteric nervous system function. Many endocrine diarrheas, such as those due to peptide-secreting tumors or hyperthyroidism,

may lead to diarrhea not only by effects on intestinal electrolyte transport but also by accelerating intestinal motility. Conversely, slow intestinal transit may lead to a secretory diarrhea by promoting bacterial overgrowth in the small intestine. Excess bacteria in the small intestine disrupt digestion and may alter electrolyte transport (e.g. diabetes mellitus and scleroderma).

Exudation Disruption of the integrity of the intestinal mucosa due to inflammation and ulceration (bacillary dysentery, ulcerative colitis) results in discharge of mucus, proteins, and blood into the bowel lumen. In such conditions the colonic absorption of water and electrolytes is severely impaired.

COMPLEX DIARRHEA

Rather than being produced by a single pathophysiologic mechanism (osmotic or secretory), most diarrheas are complex, and are produced by a combination of mechanisms. For example, a patient with malabsorption syndromes might have diarrhea because of the osmotic effects of unabsorbed carbohydrates and secretory diarrhea because of inhibition of colonic absorption by the unabsorbed long-chain fatty acids.

CLINICAL CLASSIFICATION

Clinically, it may be useful to classify diarrhea: by time course (acute vs. chronic), by volume (large vs. small), by pathophysiology (secretory vs. osmotic), by epidemiology, and by stool characteristics (watery vs. fatty vs. inflammatory).

ACUTE VERSUS CHRONIC

Acute diarrheas (<4 weeks) usually are due to infections, most of which are self-limited or are easily treatable by antibiotics. Although there are a few infectious agents that cause prolonged diarrhea in immunocompetent individuals (such as *Giardia lamblia* or *Yersinia* spp.), chronic diarrhea is usually due to some other cause.

LARGE-VOLUME VERSUS SMALL-VOLUME STOOLS

Normally rectum and sigmoid colon functions as a storage reservoir. When this reservoir capacity is compromised by inflammatory or motility disorders involving the left colon, frequent small-volume bowel movements ensue. If the source of the diarrhea is upstream in the right colon or small bowel and if the rectosigmoid reservoir is intact, bowel movements are fewer, but larger. Thus, frequent, small, painful stools may point to a distal site of pathology, whereas painless large-volume stools suggest a right colon or small bowel source. The daily total stool output may also provide etiologic hints. Irritable bowel syndrome often results in normal or only slightly elevated 24-hour stool weights, whereas diarrheas due to etiologies like

VIPomas or medullary thyroid carcinoma may produce fecal output of greater than 1 L/day.

OSMOTIC VERSUS SECRETORY

Because osmotic diarrhea is due to ingestion of some poorly absorbed substance, it abates with fasting. Secretory diarrheas typically continue during fasting, although stool output may decrease somewhat due to reduced endogenous secretions. Measurement of stool electrolyte concentration, osmolality and osmotic gap will also help differentiate between secretory and osmotic diarrhea.

WATERY VERSUS FATTY VERSUS INFLAMMATORY

Watery diarrhea implies a defect primarily in water absorption due to increased electrolyte secretion or reduced electrolyte absorption (secretory diarrhea) or ingestion of a poorly absorbed substance (osmotic diarrhea). Fatty diarrhea implies defective absorption of fat in the small intestine. Inflammatory diarrhea implies the presence of one of a limited number of inflammatory or neoplastic diseases involving the gut.

(Part-II will include clinical evaluation and management of diarrhea).

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Intestinal ion transport and the pathophysiology of diarrhea

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Worldwide, diarrhea claims several million lives annually, mostly those of infants. Poverty, crowding, and contaminated water supplies all contribute. Almost all of these deaths could have been prevented with adequate fluid replacement. Although its incidence is much lower in the more affluent nations, diarrhea remains one of the two most common reasons for visits to pediatric emergency departments and is also common among the institutionalized elderly. Chronic diarrheas, while less common, often present diagnostic dilemmas and can be difficult to manage. This article will begin by reviewing the relevant physiology; then, with that as context, it will consider pathophysiology, both general aspects and specific diarrheal syndromes. Finally, rationales for management will be briefly considered. The review is amply referenced with some of the references listed at the end of the article and some at the JCI website (<http://www.jci.org/cgi/content/full/111/7/931/DC1>).

Physiology

All segments of intestine from duodenum to distal colon have mechanisms for both absorbing and secreting water and electrolytes. This review begins with a historical approach to the development of knowledge in the field, considering absorption and secretion separately, although, inevitably, the processes involved are interconnected.

Absorption

Over the last half-century, two key sets of observations have energized and organized research in this area. The first was the discovery in the 1960s that sugars (glu-

cose and galactose) and amino acids are absorbed across the small-intestinal brush border membrane via carriers that couple their movements to that of Na^+ (1). Na^+ coupling permits the organic solute to be transported uphill, i.e., from low luminal to higher cell concentration, a gradient opposite to that for Na^+ . The organic solutes then move downhill from enterocyte to blood via basolateral membrane carriers that operate independently of ion movements. As discovered subsequently, some oligopeptides, instead of being first hydrolyzed into amino acids, are absorbed intact across the intestinal brush border by a proton-coupled mechanism. This absorptive process is indirectly coupled to Na^+ transport, since the needed protons are provided by Na/H exchange, which acidifies the unstirred layer abutting the brush border membrane (2, S1). The Na^+ gradient, therefore, is the driving force for amino acid, oligopeptide, and sugar absorption. As these organic solutes are absorbed, salt is absorbed with them, and water follows osmotically — transport from enterocyte to lateral intercellular space creates a local osmotic gradient that initiates water flow. As described below, the coupled transport of Na^+ and organic solute is the theoretical basis for oral rehydration therapy in severe diarrhea.

The second key set of observations arose from studies, performed in the late 1960s on human subjects, of intestinal salt absorption in the absence of nutrients (3, 4). Segments of jejunum and ileum were perfused with solutions of varied electrolyte composition, and the appearance or disappearance of ions and water was recorded. The data suggested different transport mechanisms in jejunum and ileum. In the jejunum, NaHCO_3 is absorbed via Na/H exchange (the secreted H^+ neutralizes an equivalent amount of luminal HCO_3^-) and Cl^- movement is purely passive. In the ileum (and, as shown later, also in the proximal colon) (S2), NaCl is absorbed via equal rates of Na/H and Cl/HCO_3 exchanges.

Three Na/H exchangers (NHEs) have since been localized to intestinal brush border membranes and cloned; NHE2 and NHE3 are found in both small intestine and colon (5, S3). NHE3 appears to be quantitatively more important, since the NHE2 knockout mouse suffers gastric dysfunction but no intestinal disability (S4) whereas the NHE3 knockout mouse suffers from

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Nonstandard abbreviations used: Na/H exchanger (NHE); downregulated in adenoma (DRA); putative anion transporter 1 (PAT1); cholera toxin (CT); protein kinase A (PKA); cystic fibrosis (CF); cystic fibrosis transmembrane conductance regulator (CFTR); NHE3 kinase A regulatory protein (E3KARP); vasoactive intestinal peptide (VIP); enteric nervous system (ENS); heat-stable toxin (STa); cGMP-dependent protein kinase (PKG); oral rehydration solution (ORS).

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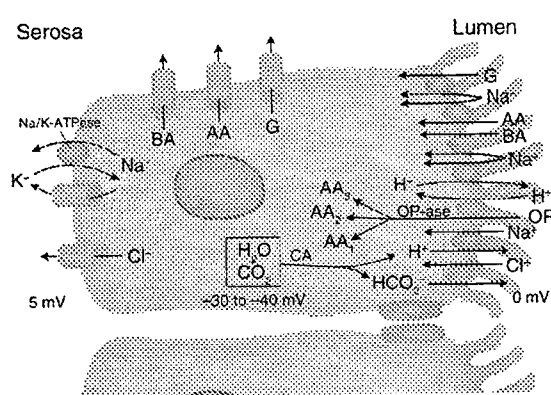


Figure 1
Ileal absorptive cell. Multiple brush border transporters couple ion influxes (Na^+ and, in one instance, H^+) to organic solute influxes or exchange one ion for another. Basolateral-membrane carriers facilitate diffusion of organic solutes and are not coupled to ion movements. Na/K -ATPase in the basolateral membrane uses energy from ATP hydrolysis to drive Na^+ extrusion and K^+ uptake (3:2 stoichiometry), both moving against their electrochemical gradients. K^+ and Cl^- channels in the basolateral membrane open in response to cell swelling and elevations of intracellular Ca^{2+} . Some of the cellular H^+ and HCO_3^- extruded in exchange for Na^+ and Cl^- is provided through the action of carbonic anhydrase (CA). Additional HCO_3^- may enter the cell through the basolateral membrane NHE1 or the $\text{Na}(\text{HCO}_3)_3$ cotransporter (not shown). The cell electric potential is 30–40 mV negative relative to the lumen, providing an electric, as well as a chemical, driving force for Na^+ entry via Na -organic solute cotransport. G, glucose or galactose; AA, amino acid (there are actually several amino acid carriers); BA, bile acid anion; OP, oligopeptide; OPase, oligopeptidase (almost all oligopeptides entering the cell intact are quickly hydrolyzed).

chronic diarrhea (6). A third, Cl^- -dependent NHE is found in crypt cells of rat distal colon (S5). The NHE first identified in intestine, NHE1, is present only in the basolateral membrane of enterocytes and is involved in HCO_3^- secretion (see below).

Two anion exchangers have also been localized to small-intestinal and colonic brush border membranes and cloned (7, S6). They have been given curious names: downregulated in adenoma (DRA) and putative anion transporter 1 (PAT1). DRA was first cloned from colonic mucosa; it was found to be downregulated in villus adenomas and carcinomas (S7) and subsequently was found to incur mutations in the rare diarrheal disorder familial chloride diarrhea (8) (see “Congenital diarrheas” below). Both DRA and PAT1 are abundant in the duodenum and present at higher density there than NHE2 and NHE3, suggesting a role in duodenal alkalization (S8). In the colon, DRA appears to predominate over PAT1 (S6).

More than two brush border ion exchangers are required, of course, for the enterocyte to engage in transcellular salt absorption. Increased turnover of the Na/K pump and the opening of Cl^- and K^+ channels are also necessary, the latter to counteract associated cell swelling, to permit serosal exit of Cl^- taken up from the lumen, and to dissipate the added uptake of K^+ through the pump. The cellular mechanisms involved are summarized in Figure 1.

Secretion

Knowledge about secretion arose from two sources: university research laboratories preoccupied with intestinal physiology, or with epithelial physiology more generally, and field stations in Asia dedicated to cholera research. As investigators moved back and forth between field stations and universities, these initially separate groups came together. Until the late 1960s, despite emerging research into cholera (see below), the gut was generally thought of as an absorptive epithelium. Rare reports of active secretion by intestinal epithelium were largely ignored (S9). In 1965, an *Annual Review of Physiology* article entitled “Secretory mechanisms of the digestive tract” did not even mention intestinal secretion (S10), whereas, in 1970, the same annual published a review devoted to intestinal secretion (S11).

Critical to cholera research were two essentially simultaneous reports in 1959, one from Calcutta (9), the other from Bombay (10), that a cell-free filtrate of a *Vibrio cholerae* culture will elicit fluid secretion when instilled into the intestines of rabbits, proving that a substance released by the bacteria (cholera toxin) is responsible. A year later, in a report on small-bowel biopsies taken from both acutely ill and convalescent cholera patients, the intestinal epithelium was demonstrated to remain intact, seemingly uninjured by the cholera organism and its toxins (11). These two reports set the stage for the investigation of cholera as an active-transport disease, or, more specifically, for exploration of the possibility that cholera toxin (CT) behaves like a lumenally active hormone, stimulating the intestinal epithelium to actively secrete electrolytes and water. However, because cholera researchers, like other gastrointestinal tract physiologists, were not yet prepared to think of the intestine as a secretory epithelium, alternative explanations were initially proposed that ultimately proved wrong.

In the late 1960s and into the 1970s, the ion transport properties of rabbit ileum were systematically examined in vitro using the isolated mucosa (stripped of muscle) mounted in so-called Ussing chambers. cAMP and agents that increase its intracellular concentration were shown to have two striking and mutually reinforcing effects on ion transport: (a) stimulation of active Cl^- secretion (“active” refers here to uphill transport, i.e., transport against an electrochemical gradient), and (b) inhibition of active, electroneutral NaCl absorption (12, S12). Coupled absorption of Na^+ and glucose was not affected. The implication of these observations for the pathophysiology of diarrhea was obvious, and these investigators quickly began a collaboration with cholera researchers to explore the possible role of cAMP in cholera in particular and diarrheal disease more generally. The collaboration soon established that CT, when added to rabbit ileal mucosa in Ussing chambers, produces the same effects as cAMP, albeit with a slower time course, and does so by stimulating adenylyl cyclase activity in enterocytes (13, 14).

Over the next decade, the individual cell membrane transporters contributing to active Cl^- secretion were

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functionally identified, giving rise to a model of general applicability to several electrolyte-secreting organs (Figure 2). cAMP, via protein kinase A (PKA), stimulates secretion by activating or enhancing the transport activities of three membrane proteins:

1. *The apical anion channel.* cAMP opens the apical anion channel (to be precise, it increases the probability of its opening), thereby initiating secretion (15). In the intestine, at least, cAMP also promotes insertion of additional channels (recruited from the ER) into the apical membrane (S13, S14).

2. *A basolateral membrane K⁺ channel.* The opening of a basolateral membrane K⁺ channel repolarizes the cell, counteracting the depolarizing effect of opening the Cl⁻ channel and thus sustaining the electrical driving force for Cl⁻ extrusion into the lumen. A cAMP-activatable K⁺ channel has recently been identified and cloned (16, S15, S16).

3. *A basolateral membrane NaK2Cl cotransporter.* The turnover of a basolateral membrane NaK2Cl cotransporter (17, S17) determines the maximal rate of serosal Cl⁻ entry (once the apical anion channel and the basolateral membrane K⁺ channel have been activated). cAMP appears to enhance cotransporter activity not via PKA-induced phosphorylation of the transport protein, but indirectly by opening the apical anion channel and thereby decreasing cell [Cl⁻], and also by enhancing cotransporter insertion into the basolateral membrane from an intracellular storage site (S17, S18).

The inherited disease cystic fibrosis (CF) develops when dysfunctional mutations occur in the gene for the cAMP-responsive apical anion channel. Accordingly, this channel, which is present in a number of tissues, has been dubbed the cystic fibrosis transmembrane conductance regulator or CFTR for short. CFTR is a member of the superfamily of ABC proteins and has a single-channel conductance of 7–10 pS and a linear current-voltage relationship (18). Studies of intestine from both humans and transgenic mice with CF show a near total absence of electrolyte secretion (19, 20), which perhaps explains why patients with CF occasionally develop intestinal obstruction (known, in neonates, as meconium ileus).

Not only is cAMP's secretory effect lost in CF, but its inhibition of electroneutral NaCl absorption is also lost, indicating a critical role for normal CFTR in the latter. The mechanism for cAMP's (and also Ca²⁺'s) inhibition of electroneutral NaCl absorption is not fully elucidated but is known to involve several proteins, including NHE3 kinase A regulatory protein (E3KARP), NHE regulatory factor (NHERF), ezrin, and α -actinin-4 (S19–S21). NHE3, DRA, and CFTR all possess affinity for a discrete binding domain on E3KARP. It has recently been proposed that CFTR, NHE3, and either DRA or PAT1 bind to one domain of E3KARP (which dimerizes in the process) (S19, S20) and that signal transduction proteins such as PKA (via ezrin) (S21) bind to a second domain. Whether PKA inhibits electroneutral NaCl absorption by activating the bound CFTR or through a more direct effect on E3KARP remains to be determined.

Regulation of absorption and secretion

Once a role for cAMP in gut electrolyte secretion was established, the hunt was on for regulatory endocrine/paracrine/neural compounds. Two classes of compounds emerged: those that stimulate active secretion and inhibit active absorption, and those with the opposite effects (Table 1). The former group includes four kinds of agents: (a) neurotransmitters, including vasoactive intestinal peptide (VIP), acetylcholine, substance P, and the nucleotides ATP and UTP; (b) the paracrine agents serotonin and neurotensin, which are released by endocrine (enterochromaffin) cells in the intestinal epithelium; (c) agents released by inflammatory cells, including mainly prostaglandins and leukotrienes but also platelet-activating factor (which causes other inflammatory cells to release prostaglandins) (S22), histamine, and serotonin; and (d) guanylin, a lumenally active peptide released into the gut lumen by goblet cells (see "Toxicogenic *E. coli*" below).

Among these compounds, cAMP mediates the action of VIP, UTP/UDP, and prostaglandins (at high concentration). The action of guanylin is mediated by cGMP (see below). For all of the others (including lower concentrations of prostaglandins), activation of their receptors increases intracellular Ca²⁺ and activates PKC. The Ca²⁺ increase opens a basolateral K⁺ channel dif-

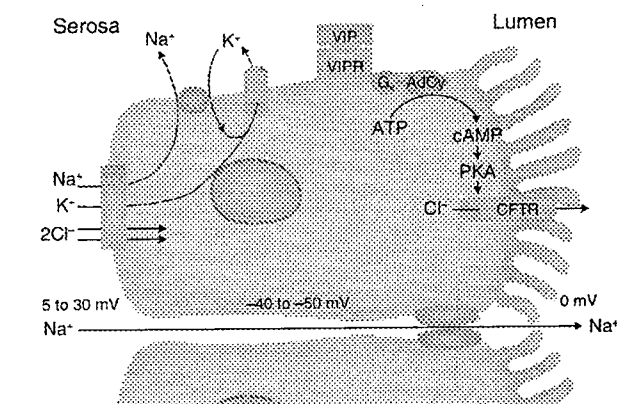


Figure 2
Intestinal Cl⁻ secretion. CFTR, the apical anion channel, mutations in which cause cystic fibrosis, is opened by PKA-catalyzed phosphorylations (its activity can also be enhanced through protein kinase G- and PKC-induced phosphorylations). The VIPR receptor is a G protein-linked activator of adenylyl cyclase (AdCy). The electroneutral NaK2Cl cotransporter facilitates the coupled movement of these ions across the basolateral membrane. The drop in cell [Cl⁻] that follows opening of the apical anion channel enables an inward flux of the three ions. Cotransporter activity also increases in response to cell shrinkage. When CFTR is opened, the resulting anion efflux depolarizes the cell's electric potential. The repolarization required to maintain secretion is accomplished through the opening of a cAMP-sensitive, Ca²⁺-insensitive K⁺ channel in the basolateral membrane. During secretion, the cell electric potential is 40–50 mV negative and the serosal potential is 5–30 mV positive, both relative to the lumen (the serosal potential tends to be closer to 5 mV in the small intestine and 30 mV in the distal colon). The transmural potential difference drives Na⁺ movement into the lumen through the tight junctions via the lateral intercellular spaces.

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Table 1
Local control of intestinal ion transport

Agonist	Source	Target cell(s)	Intracellular mediator(s)	Secondary mediator(s) of transporting epithelial cell effects
Secretory/Antiabsorptive stimuli				
VIP (S91)	Neural	Epith	cAMP	
ACh (S92, S93)	Neural	Epith, neural, mes	Ca ²⁺ , PKC	Eicos, ? VIP
SP (S94-S96)	Neural	Neural, mast, epith	Ca ²⁺ , PKC	Histamine, eicos
ATP/ADP (S97)	Neural	Mes, epith	Ca ²⁺ , PKC	Eicos
UTP/UDP (S97)	Neural	Mes, epith	cAMP	Eicos
5-HT (S98, S99)	EC, mast cell	Neural, epith	Ca ²⁺ , PKC	VIP and ACh
NT (S94, S100)	EC	Neural	Ca ²⁺ , PKC	Eicos, adeno, VIP, and ACh
Guanylin (39)	Goblet cells	Epith	cGMP	
PGs (13, S101)	Mes	Epith, neural	Ca ²⁺ , PKC, cAMP	
LTs, pre-LTs (S28, S102)	Mes	Epith, neural	Ca ²⁺ , PKC	
PAF (S22, S103)	Mes	Mes	Ca ²⁺ , PKC, cAMP	Eicos
Histamine (S104, S105)	Mast cell	Mes, neural	Ca ²⁺ , PKC, cAMP	Eicos, VIP, ACh
Bradykinin (S106)	Vascular	Mes	Ca ²⁺ , PKC, cAMP	Eicos
Adenosine (S107-S111)	Epith, lumen	Epith, mes	A1 R: Ca ²⁺ , PKC A2 R: cAMP	PGs (not LTs)
Endothelin-1 (S112)	Vascular	Mes	Ca ²⁺ , PKC, cAMP	PGs
Antisecretory/Proabsorptive stimuli				
Norepi (22, S32, S113)	Neural	Epith, neural	Activate G _i	
NPY (S32, S114-S117)	Neural, EC	Epith, neural	?	? Norepi
SST (S26, S118, S119)	EC, neural	Epith, neural	Activate G _i (SST R2)	
Enkeph (S120, S121)	Neural	Epith, neural	?	
12-HETE (S27)	Mes	Epith	Block BL K ⁺ channel	

Epith, epithelial; ACh, acetylcholine; mes, mesenchymal cell (fibroblast, neutrophil, eosinophil, lymphocyte, mast cell, etc.); SP, substance P; eicos, eicosanoids (prostaglandins, leukotrienes, and their precursors); 5-HT, 5-hydroxy-tryptamine (serotonin); EC, enterochromaffin or epithelial endocrine cell; NT, neurotensin; adeno, adenosine; PG, prostaglandin; LT, leukotriene; PAF, platelet-activating factor; R, receptor; norepi, norepinephrine; G_i, inhibitory G protein; NPY, neuropeptide Y; SST, somatostatin; enkeph, enkephalins (opioids); 12-HETE, 12-hydroxyeicosatetraenoic acid; BL, basolateral. Numbers in parentheses indicate references.

ferent from that opened by cAMP (21). Based on studies with other tissues, PKC activation may either directly open the CFTR anion channel or heighten its sensitivity to PKA (S23-S25).

A striking difference between the effects of cyclic nucleotide-dependent secretagogues and Ca-dependent ones is evident in vitro. The former cause persistent secretion, whereas the latter cause transient secretion and the swift development of tachyphylaxis. This difference may be less pronounced in vivo, since the relevant effectors are quickly removed by hydrolysis and blood flow, perhaps thereby preventing or reversing tachyphylaxis.

The group of compounds that both inhibit active secretion (HCO₃⁻ as well as Cl⁻) and enhance active absorption includes norepinephrine (via α₂-receptors), neuropeptide Y, enkephalins, all neurotransmitters, and somatostatin, both a neurotransmitter and a paracrine agent (S26). Among leukotriene precursors, 12-hydroxyeicosatetraenoic acid is antisecretory, blocking the basolateral membrane Ca²⁺-activated K⁺ channel (S27). Other leukotriene precursors are secretory stimuli (S28).

With a multitude of locally produced compounds providing bidirectional regulation of intestinal ion transport, it is not surprising that the system can be fine-tuned for a variety of circumstances. In the small intestine, so-called basal conditions may in fact represent a low rate of secretion (S29). Attempting to outline the complex, multifaceted relationships among epithelial ion-transporting

cells, epithelial endocrine cells, mesenchymal cells (fibroblasts, mast cells, neutrophils, T and B lymphocytes, eosinophils, etc.), and nerves is beyond the scope of this review. Some of the complexity can be gleaned from Table 1. A few points can be emphasized as follows.

The enteric nervous system (ENS) consists of two plexi, the myenteric (between the circular and the longitudinal smooth muscle layers) and the submucosal, along with their interconnections. Both plexi contain so-called secreto-motor nerves that release agonists with either direct or indirect effects on epithelial ion transport. In the isolated (muscle-stripped) mucosal preparation, the submucosal plexus is usually retained, but not the myenteric. That the former alone profoundly influences ion transport is evident from the effect of adding tetrodotoxin to this preparation (S30).

VIP and acetylcholine are the predominant transmitters with direct epithelial secretory and/or antiabsorptive effects released by secreto-motor nerves; acetylcholine also acts on other neurons and on mesenchymal cells.

The mast cell is a key mediator for epithelial-neural interactions in allergic reactions and some parasitic infections (S31). Activation of mucosal mast cells by IgE or a variety of agonists including substance P, acetylcholine, and neurotensin causes release of histamine, serotonin, rat mast cell protease II (which appears to enhance absorption of macromolecules), prostaglandins, leukotrienes, and platelet-activating factor.

Norepinephrine, the predominant antisecretory/proabsorptive agonist in the intestines, activates α_2 -receptors on both enterocytes (22) and nerves (S32, S33). The neural effect is inhibitory, blocking release of secretion-inducing neurotransmitters. The direct epithelial effect (and perhaps also the neural effect) is exerted, in part at least, through inhibition of adenylyl cyclase. Adrenergic fibers are present in the submucosal plexus.

Other transport and permeability properties

Barrier function. In addition to transporting ions, nutrients, and water, the intestinal epithelium, consisting of both enterocytes and their encircling tight junctions (zona occludens), functions as a barrier that restricts the flow of luminal contents into the blood and lymphatics and vice versa. In the small intestine, tight junctions are, on the average, of the low-resistance type, meaning that most of the passive permeability of the epithelium to small monovalent ions and water resides in these junctional complexes (tight junctions in villi have higher resistance than do those in crypts) (S34). Colonic intercellular junctions are tighter, their resistance increasing steadily from proximal to distal portions. Although these junctions, which comprise a number of discrete proteins, are extracellular, their permeability properties are regulated by intracellular structures, especially actin filaments (23).

Osmolarity of intestinal contents. Unlike renal tubules, the intestines neither dilute nor concentrate their contents, the osmolarity of which, except in duodenum and proximal jejunum shortly after eating, is the same as plasma osmolarity (24). Although it may seem counterintuitive, the osmolarity of ions and molecules in stool water is also identical to plasma osmolarity (S35).

Other ion transport mechanisms and their geography. Mechanisms vary both longitudinally, along the length of the intestines, and horizontally, along the crypt-to-villus or crypt-to-surface cell axis.

Longitudinally, differences exist in Na^+ entry mechanisms, sites of HCO_3^- secretion, and sites of active K^+ transport. Na^+ crosses small-intestinal and colonic brush borders via Na/H exchange and, in the small bowel, also by Na -organic solute cotransport. In the distal colon, luminal Na^+ is also absorbed via an aldosterone-sensitive Na^+ channel that belongs to the same molecular family as does the channel in the distal renal tubule (S36). In hyperaldosteronemic states (dehydration, liver disease, and heart failure), colonic Na^+ absorption (and therefore also Cl^- and water absorption) is enhanced.

HCO_3^- is absorbed in the jejunum (via Na/H exchange) and secreted in the duodenum, ileum, and colon. Its secretion can be explained in part by Cl/HCO_3^- exchange (when it is not offset by Na/H exchange), but most intestinal HCO_3^- secretion appears to occur via CFTR, which is permeable to both HCO_3^- and Cl^- ; the relative rates of secretion of HCO_3^- and Cl^- appear to be determined by cell-to-lumen concentration differences and by their relative conductivity through CFTR (25). At the enterocyte's basolateral membrane, HCO_3^- entry involves both Na/H exchange and $\text{Na}(\text{HCO}_3)_3$ cotransport (S37-S39).

Active absorption and secretion of K^+ both occur in the colon, but neither occurs in the small intestine, where K^+ movement is strictly diffusional. K^+ is actively absorbed in distal colon via an H/K -ATPase homologous to the gastric H/K -ATPase responsible for acid secretion and is actively secreted throughout the colon via apical K^+ channels (26, S40, S41). K^+ absorption is enhanced by Na^+ and K^+ depletion and inhibited by cAMP. K^+ secretion is enhanced by chronic dietary K^+ loading and by cAMP.

Horizontally, the anatomic sites for secretion and absorption have traditionally been thought to differ, secretion occurring in crypts and absorption in small-intestinal villi and colonic surface cells. This undoubtedly overreaching generalization is consistent with some studies but not others. In support, (a) hypertonic disruption of small-intestinal villus cells does not reduce CT-induced secretion (27); (b) in distal colon, intracellular microelectrode recordings have shown appropriate electrical responses to secretory stimuli only in crypt cells, not in surface cells (S42, S43); and (c) almost all CFTR molecules have been localized to small-intestinal and colonic crypts by immunofluorescence and in situ hybridization (28, 29). Intriguingly, however, occasional villus cells, representing 3% of the total, immunostain positive for CFTR (30). These cells, which superficially resemble neighboring villus cells but lack brush border disaccharidases, may be secretory. Alternatively, they may represent a high-conductance route for passive Cl^- movement.

On the other hand, microperfusion of individual crypts dissected from distal colon has demonstrated base-line absorption and agonist-inducible secretion (31). Does this dual functionality also apply to small-intestinal crypts? Probably not. Colonic crypts are much larger, their cells constituting most of the epithelium; in contrast, small-intestinal crypt cells normally constitute only about 20% of the epithelium. Furthermore, an apical NHE has not been found in small-intestinal crypt cells (32, S44); an apical anion exchanger is present (32), but it alone cannot enable fluid absorption.

Not all investigators will agree (S45, S46), but the following conclusions seem best supported by the evidence: (a) in small intestine, most, but conceivably not all, secretion arises from crypts, and all, or nearly all, absorption arises from villi; and (b) in colon, crypts display both absorption and secretion, but surface cells only absorb. In distal colon, Na^+ channel-mediated absorption is confined to surface cells (S47).

Due to this at-least partial separation of absorptive and secretory functions, the latter predominate in diseases that selectively damage villar or surface epithelium (e.g., enteric infection, inflammatory bowel disease, celiac sprue). This predominance is further accentuated in celiac disease, because crypt hypertrophy also develops.

Table 2, which lists the various cell membrane transport proteins that collectively account for the intestinal tract's absorptive and secretory processes for electrolytes, provides a somewhat cryptic summary of much of the above. One entry in the table is not referred to above: ClC-4 , a second apical Cl^- channel in mouse and

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Table 2
Intestinal transport proteins

Transport process	Ions transported	Species	Location		Associated gene defect (m or h)
			A/BL	Horizontal/vertical distribution	
NaCl abs	Na/H	NHE2 (S, S3, S4)	A	SI, DC (V and SC > Cr)	Gastric atrophy (m)
		NHE3 (S, 6, S3, S4)	A	SI; PC (V, SC >> Cr)	Chronic diarrhea (m)
		Cl-dep. NHE (S5)	A	DC (Cr)	
	Cl/HCO ₃	dra (7, 8, S8)	A	Duod, ileum, C (V and Cr)	Familial Cl- diarrhea (h)
		PAT1 (S6)	A	Duod, ileum > C	
Na ⁺ abs	K ⁺	High G; Ca ²⁺ , stretch-activated (21, S122-S125)	BL	Throughout	
	Cl ⁻	ClC-2; PKC-activated (S126, S127)	BL	Throughout	
	Na ⁺	ENa (~4 pS) (S36, S47)	A	DC (SC)	
Cl ⁻ secr	K ⁺	High G; Ca ²⁺ , stretch-activated	BL		
	Cl ⁻	CFTR (~7 pS) (15, S13, S14)	A	Throughout (cr >> V, SC)	Cystic fibrosis (m, h)
		ClC-4 (S49)	A	Same as for CFTR	
	K ⁺	High G (200–300 pS); Ca ²⁺ , stretch-activated	BL	Throughout	
HCO ₃ ⁻ abs	Na ⁺ , K ⁺ , Cl ⁻	Low G, cAMP-activated; (16, S15, S16, S121, S125, S126)	BL	Throughout (cr >> V)	
		NKCC1 (17, S17, S18)	BL	Throughout	
	Na ⁺ , H ⁺	NHE2, NHE3	A	Jej (V)	
	K ⁺	High G (200–300 pS); Ca ²⁺ , stretch-activated	BL		
HCO ₃ ⁻ secr	Cl/HCO ₃	Dra, PAT1	A	Duod, ileum, C (V, SC, cr)	
	HCO ₃ ⁻	CFTR (25)	A	Duod, ileum, C (cr >> V)	
	Na/H	NHE1	BL	Throughout (cr, V, SC)	
	Na(HCO ₃) ₃	NBC1 (S37, S38)	BL	Duod, PC	
K ⁺ abs	K/H	H ⁺ /K ⁺ -ATPase (26)	A	DC	
	K ⁺	High G; Ca ²⁺ , stretch-activated	BL		
K ⁺ secr	K ⁺	High G, aldo-activated (S40, S41)	A	C (cr)	
	Na ⁺ , K ⁺ , 2Cl ⁻	NKCC1	BL	Throughout	
	Na/K	Na/K-ATPase	BL	Throughout	

Since the action of Na/K-ATPase on the basolateral membrane is central to all of these ion transport processes, its involvement is assumed without mention, except in the instance of K⁺ secretion, where it serves as a K⁺-loading mechanism. A, apical membrane; BL, basolateral membrane; m, transgenic mouse; h, human; abs, absorption; SI, small intestine; PC, proximal colon; V, villus; SC, colonic surface cell; Cr, crypt; dep., dependent; DC, descending colon; dra, downregulated in adenoma; duod, duodenum; C, colon; G, conductance; ENa, Na⁺ channel; secr, secretion; aldo, aldosterone.

human intestine that belongs to the family of ClC anion channels (S48) and that colocalizes with CFTR (S49). It is activated by depolarization but the effects, if any, of intracellular mediators or extracellular regulators on its activity are unknown. Whether its variable expression can explain the far more severe intestinal disease that occurs in CF knockout mice than in humans with CF is unclear. ClC-4 has been detected in both murine and human intestine. Only minimal secretion, which is not increased by traditional effectors, has been observed in CF intestinal preparations. Since CF gene mutations generally interfere with CFTR traffic to the apical membrane (S50), and since normal CFTR is a regulator of other membrane transporters and ion channels, apical placement of ClC-4 may also be affected in CF. It will be interesting to look at ClC-4 expression in CF intestine.

Pathophysiology: general aspects

Patients may use the word diarrhea for increases in stool mass (in adults, up to 250 g/d is considered normal), stool liquidity, or stool frequency. The first criterion truly defines diarrhea, but patients rarely provide quantitative information on their daily stool output. The

other two criteria suggest the first, but occasionally stool frequency or liquidity increases without an appreciable increase in stool mass. Most such instances constitute a purely functional disorder known as irritable bowel syndrome, which is responsible for at least 25% of all visits to gastroenterologists. Although moderate increases in stool mass (500–1,000 g/d) need medical attention if they persist, this review is mainly concerned with more severe diarrheas — those that can lead to dehydration.

Two caveats about diarrhea are worth stressing. First, significant malabsorption of nutrients does not always cause diarrhea, since the normal adult colon's reabsorptive capacity is about 5 l/d. Second, patients may develop profuse diarrhea in the absence of nutrient malabsorption or even histologically evident mucosal damage or inflammation, as, for example, when the small intestine's immense secretory capacity is triggered by a bacterial enterotoxin.

Diarrheal driving forces

Osmosis, active secretion, exudation, and altered motility can all drive diarrhea. Specific diarrheal illnesses often involve more than one of these forces.

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Osmotic diarrhea. When poorly absorbable, low-molecular weight aqueous solutes are ingested, their osmotic force quickly pulls water and, secondarily, ions into the intestinal lumen. Individuals with normal gut function will develop osmotic diarrhea when they ingest large amounts of poorly absorbable solutes, such as lactulose (if they are being treated for hepatic encephalopathy), sorbitol (if they continually chew sugar-free gum), or Mg^{2+} (if they take certain antacids or bowel purgatives).

Osmotic diarrhea can also develop when an ordinarily absorbable nutrient is ingested by an individual with an absorptive defect, for example, lactose by someone with congenital lactase deficiency, or carbohydrate by someone with gluten-sensitive enteropathy (celiac disease). Maldigestion, as seen in pancreatic insufficiency, may cause osmotic diarrhea of colonic origin: unabsorbed carbohydrates and lipids, on reaching the colon, are hydrolyzed by bacteria into short-chain organic acids, the quantity of which may overwhelm the colon's capacity for their absorption.

Secretory diarrhea. Diarrhea resulting from overstimulation of the intestinal tract's secretory capacity can develop in "pure" form (e.g., cholera) or as a component of a more complex disease process (e.g., celiac disease, Crohn disease). "Pure" secretory diarrhea is characterized by (a) large stool volumes (which can exceed 1 liter per hour in well hydrated adults), (b) absence of red or white blood cells in the stool, (c) absence of fever or other systemic symptoms (except those due to dehydration), (d) persistence of diarrhea with fasting (volume may diminish, however), and (e) lack of excess osmotic gap in stool electrolytes. Osmotic gap (OG) is defined as follows: $OG = 290 - 2\{[Na^+] + [K^+]\}$, where 290 is the assumed osmolality of blood plasma. A gap greater than 50 mOsm is considered abnormal (S51); the normal gap is made up of Mg^{2+} , Ca^{2+} , NH_4^+ , and perhaps organic cations. The pattern of stool electrolytes in patients with acute cholera shows Na^+ , K^+ , and Cl^- concentrations not very different from those in plasma and an HCO_3^- concentration somewhat higher than in plasma. In contrast, normal stool shows low $[Na^+]$ and high $[K^+]$ concentrations, due mainly to the colon's reabsorption of Na^+ and secretion (both active and passive) of K^+ ; and a low $[Cl^-]$ concentration, due to the replacement of $[Cl^-]$ by short-chain organic acid anions generated by colonic bacteria. Normally, $[HCO_3^-]$ concentration is similar to that in plasma.

A number of secretory stimuli can cause diarrhea. These include bacterial enterotoxins, hormones generated by endocrine neoplasms, dihydroxy bile acids, hydroxylated fatty acids, and inflammatory mediators. In the absence of vigorous fluid replacement, marked dehydration leading to vascular collapse can occur, as can severe losses of K^+ and HCO_3^- .

Exudative diarrhea. If the intestinal epithelium's barrier function is compromised by loss of epithelial cells or disruption of tight junctions, hydrostatic pressure in blood vessels and lymphatics will cause water and electrolytes, mucus, protein, and sometimes even red and white cells to accumulate lumenally (e.g., ulcerative colitis, shigellosis, intestinal lymphangiectasia). If the con-

dition is chronic, the continuing protein loss can lead to hypoalbuminemia and hypoglobulinemia.

Diarrhea resulting from motility disturbances. Both increases and decreases in gut motility can lead to diarrhea. Examples of the former are thyrotoxicosis and opiate withdrawal. Decreases in effective motility in the small intestine due to large diverticula, smooth muscle damage (scleroderma, dermatomyositis, amyloidosis, muscular dystrophy, or radiation injury), or autonomic neuropathy (diabetic, idiopathic) can result in bacterial overgrowth. As specified below, bacterial overgrowth can lead to diarrhea.

Pathophysiology of acute diarrheas

Enteric infection with enterotoxin-producing bacteria

Summarized here is information on three bacterial enterotoxins produced by two enteric organisms, *V. cholerae* and *Escherichia coli*. In fact, similar toxic peptides are produced by *Yersinia* (S52) and *Salmonella* (S53) and perhaps other enteropathogenic bacteria.

***V. cholerae*.** We are in the midst of a worldwide pandemic of cholera, due to biotype El Tor, that apparently began in the 1960s (S54). The disease is now or has been recently prevalent in India, Bangladesh, Pakistan, Indochina, Indonesia, Afghanistan, Africa, South America, and Mexico. Although it is rare in the US, 61 proven cases were reported to the Centers for Disease Control from 1995–2000 (S55). More than half were acquired during travel abroad, and most of the others were acquired from undercooked imported seafood. The organism appears to inhabit estuaries (in a form that is difficult to culture) and to spread to human populations under conditions of contaminated water supplies, inadequate sewage disposal, crowding, and malnutrition. Wars and earthquakes are inciting factors.

Every medical student now learns the mechanism of action of CT, the *V. cholerae* enterotoxin. CT is an 84-kDa protein consisting of a dimeric A subunit and five identical B subunits (Figure 3). The A subunit consists of two peptides linked by an S-S bond, the larger, A1, containing the toxic activity. Each of the B subunits binds tightly to the GM1 ganglioside abundant in the intestinal brush border membrane (33); binding is so tight that only the normal 5- to 7-day turnover of the epithelium eliminates toxicity. The toxin's A1 subunit, which is endocytosed following toxin binding to GM1 (S56), covalently modifies the α subunit of G_s , the adenylyl cyclase-stimulating G protein. More specifically, the endocytosed A1 subunit catalyzes covalent bonding of adenosine diphosphoribose from NAD to the α subunit of G_s ($G_{s\alpha}$), inhibiting the intrinsic GTPase activity of $G_{s\alpha}$ thereby preventing self-inactivation of its adenylyl cyclase-stimulating activity (34, 35).

V. cholerae produces an additional physiologically active peptide, zona occludens toxin, that increases the permeability of tight junctions (S57); however, its relevance to diarrhea is unclear.

Toxigenic *E. coli*. Toxigenic *E. coli*, which proliferate in institutional settings and commonly infect American

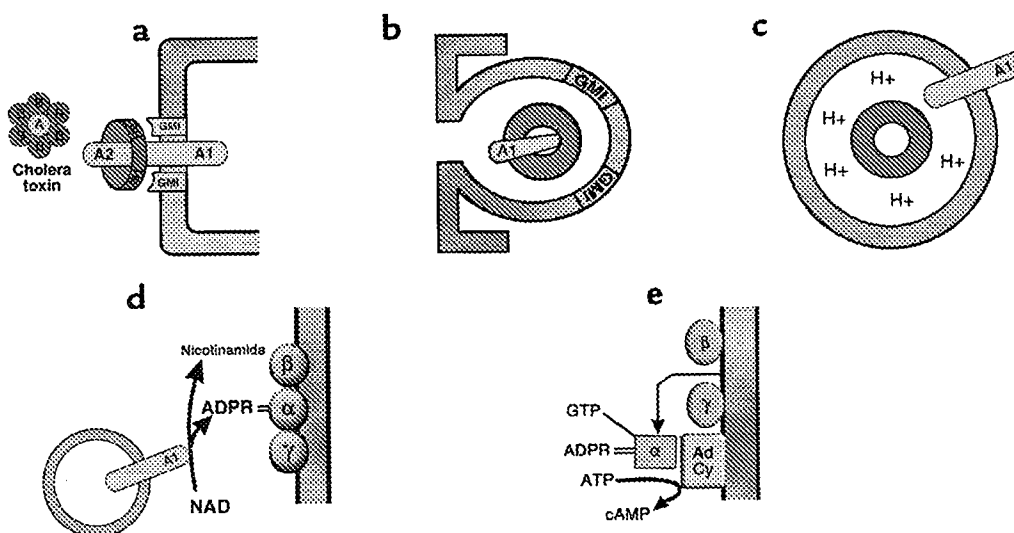


Figure 3

Cellular mechanism of action of cholera toxin. (a) The toxin, through its B subunits, which encircle the enzymically active A subunit, binds to the ubiquitous glycolipid membrane receptor, the monosialoganglioside GM1. (b) The entire complex is endocytosed by both clathrin-dependent and clathrin-independent means. (c) Inwardly directed proton pumps acidify the CT-containing endocytic vesicle, causing the toxin subunits to dissociate. Then the enzymically active A1 peptide is inserted into the vesicle membrane, with its catalytic site exposed to cytoplasm. (d) The A1 peptide is an ADP-ribosyltransferase that cleaves NAD into adenosine diphosphoribose (ADPR) and nicotinamide and covalently bonds the former to the α subunit of the G_s adenylyl cyclase-stimulatory G protein. (e) The intrinsic GTPase activity of the ADPR-modified α subunit of G_s is markedly inhibited, allowing GTP to remain bound to it; the $G_s\alpha$ -GTP complex separates from its membrane mooring to $G_s\beta$ and $G_s\gamma$, attaching to and activating adenylyl cyclase (AdCy).

travelers abroad (traveler's diarrhea), elaborate at least two secretion-stimulating enterotoxins. The first, heat-labile toxin, is a high-molecular weight peptide that is immunologically related to CT and has the same cellular mechanism of action (S58). The second, heat-stable toxin (STa), is a cysteine-rich, low-molecular weight, heat-stable peptide (S59) that activates guanylate cyclase in the intestinal epithelium (36), thereby activating cGMP-dependent protein kinase (PKG), which, like PKA, can open the CFTR anion channel (37) (Figure 4). Twelve years ago, the brush border receptor for STa was cloned and identified as a guanylyl cyclase, which is activated upon STa binding (38). The existence in mammalian intestine of an enzymatically active receptor for a bacterial enterotoxin raised the question of whether an endogenous ligand for this receptor, one with structural homology to STa, also exists. This question was answered about a year later when a low-molecular weight peptide that mimics STa action and has structural homology to STa was extracted from rat intestinal contents and dubbed "guanylin" (39). This luminally active hormone is, like STa, rich in cysteine (although its four cysteines are two less than in STa). Since it is synthesized in and secreted from goblet cells, and its synthesis is enhanced by oral salt loading, it may play roles in both fluid homeostasis and the moistening of goblet cell mucoid secretions (S60, S61).

As an aside, a close homolog of guanylin, known as uroguanylin, also originates in the gut epithelium and is secreted into the blood by enterochromaffin cells (S62). It is a diuretic (at least in the kidneys of rodents) and may explain why an oral salt load induces a greater

diuresis than the same salt load given intravenously.

Role of the ENS in enterotoxin-induced secretion. Although the direct effects of CT, heat-labile toxin, and STa on the ion-transporting cells of the intestinal epithelium would seem sufficient to explain the resulting secretion (and in the *in vitro* mucosal preparation they are sufficient), there is ample evidence from *in vivo* experiments that the ENS also plays a role — in the mind of some, a dominant role. When segments of mammalian small bowel are perfused with toxin-containing solutions, most of the resulting fluid transport changes can be reversed with neural poisons such as tetrodotoxin and hexamethonium (40). Recent experiments with a specific VIP antagonist demonstrate that much (but not all) of the secretion induced in these enterotoxin perfusions is mediated by VIP (41).

The sequence of events, summarized in Figure 5, seems to be as follows. The enterotoxins attach to endocrine (enterochromaffin) cells on the villus surface of the intestinal epithelium, causing an increase of cAMP or cGMP. cAMP or cGMP triggers these cells to release serotonin, neurotensin, and possibly other peptides into the subepithelial space. These paracrine mediators set in motion a secretory neural reflex that involves (a) afferent enteric neurons that begin subepithelially and end in the myenteric plexus, (b) interneurons that connect the myenteric to the submucosal plexus, and (c) efferent neurons in the submucosal plexus that release VIP (and probably additional secretory stimuli) near the basal surface of the epithelium. The interneuronal effect appears to be mediated by acetylcholine and/or substance P. Finally, the VIP (and

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perhaps another agonist) attaches to receptors on both villus and crypt enterocytes, activating adenylyl cyclase and inducing cAMP-mediated alterations of ion transport (inhibition of absorption in villus cells and stimulation of secretion in crypt cells).

One caveat is that the role of the ENS in enterotoxin-induced secretion may be more striking in the animal studies than in the disease itself; in segmental perfusions, enterotoxin does not penetrate into the crypts, whereas, in cholera patients, a direct effect of CT on crypt cells is likely since *Vibrio* organisms proliferate in the crypts as well as elsewhere in the gut lumen.

Other enteric infections

Common bacterial causes of enteritis and/or colitis are *Shigella*, *Salmonella*, *Yersinia*, enteroinvasive *E. coli*, *Aeromonas*, and *Campylobacter*. These organisms invade the epithelium and multiply intracellularly, damaging the surface epithelium and causing inflammation. Diarrhea is due both to the epithelial damage (exudation and decreased absorptive capacity) and to the action of inflammatory mediators.

Some viruses also invade the intestinal epithelium, causing enterocyte destruction, inflammation, and a temporary sprue-like syndrome (partial villus atrophy and crypt hypertrophy). These include rotavirus (worldwide, probably the most common cause of diarrhea in infants), certain adenoviruses, and the so-called Norwalk-like agents (S63). These last agents have recently caused a flurry of brief episodes of vomiting and diarrhea among passengers on cruise ships (S64).

Some enteric bacteria produce cytolytic toxins that destroy epithelial cells, interfering with absorption and causing inflammation. These include *Shigella dysenteriae* (Shiga toxin), a protein synthesis inhibitor; *Clostridium difficile*, whose A toxin alters cytoskeletal structure, and whose proliferation is induced by broad-spectrum antibiotics; *Vibrio parahaemolyticus*; *Clostridium perfringens*; and enteroadherent and enterohemorrhagic *E. coli* (S65–S68). Most of these bacterial toxins cause breakdown of the cytoskeleton through ADP ribosylation or glucosylation of proteins from the Rho family, which regulate actin polymerization.

Cryptosporidia, a unicellular parasite, caused an epidemic of diarrhea in Milwaukee ten years ago through contamination of the city water supply (S69). Persistent cryptosporidial enteritis can occur in immunocompromised individuals. *Cryptosporidia* gain entry to the apical cytoplasm of enterocytes by first fusing with the enterocyte microvillus membrane (S70, S71). Heavy infestation with *Giardia lamblia* leads to disruption of the enterocyte microvillus membrane, which, in turn, leads to malabsorption and diarrhea (S72).

Acute T cell activation

Parenteral administration of cytolytic antibodies to T cell surface antigens causes massive acute, but short-lived, diarrhea, as seen in humans treated for rejection of transplanted organs and, experimentally, in mice (S73). The critical cytokine released in these circumstances appears to be TNF- α , presumably because it

causes the massive release of prostaglandins and other secretory stimuli.

Pathophysiology of chronic diarrheas

Congenital diarrheas

Two rare congenital autosomal-recessive defects in intestinal fluid absorption are associated with diarrhea: congenital chloride diarrhea, which results from a defect in brush border Cl/HCO₃ exchange (see above), and congenital sodium-secretory diarrhea, which results from an as-yet undefined defect in brush border Na/H exchange (8, S74). In both cases, nutrient absorption is normal. Also in both, abnormal fluid absorption begins in utero, manifesting itself as maternal polyhydramnios. Congenital chloride diarrhea has been more extensively reported, with clusters of cases found in Finland, Saudi Arabia, Kuwait, and Poland and scattered cases elsewhere (8). In this syndrome, stool pH is acidic, and metabolic alkalosis develops. Similar acid-base changes are seen in carbohydrate malabsorption and Zollinger-Ellison syndrome. In contrast, in most severe diarrheas, stool pH is alkaline, and metabolic acidosis develops.

Several other rare congenital defects in nutrient absorption or in intestinal epithelial structure can also cause neonatal diarrhea (S75): congenital sucrase-isomaltase deficiency; congenital glucose-galactose malabsorption; lysinuric protein intolerance, a defect in dibasic amino acid transport across the enterocyte basolateral membrane; acrodermatitis enteropathica, a defect in zinc absorption; and microvillus inclusion

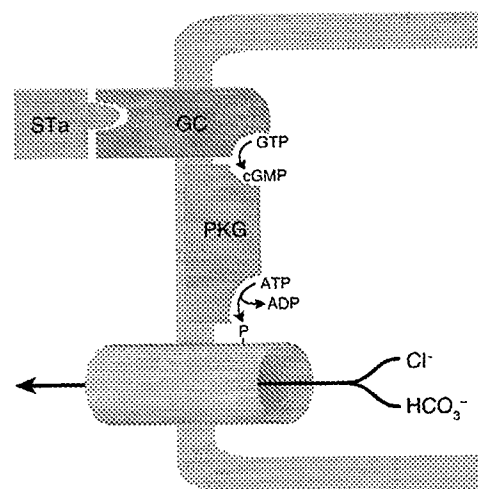


Figure 4

Cellular mechanism of action of heat-stable *E. coli* enterotoxin (STa). Luminal toxin binds to and activates guanylyl cyclase C (GC), a brush border enzyme with a high rate of expression in both small intestine and proximal colon. The resulting increase in [cGMP] activates membrane-bound protein kinase G (PKG), which, in turn, opens neighboring anion channels or inhibits neighboring Na/H and Cl/HCO₃ exchangers (not shown). Guanylin, a mammalian homolog of STa, is the physiologic agonist for the STa-sensitive GC. It is secreted into the lumen by goblet cells. In the duodenum, its action results in an HCO₃⁻-rich secretion.

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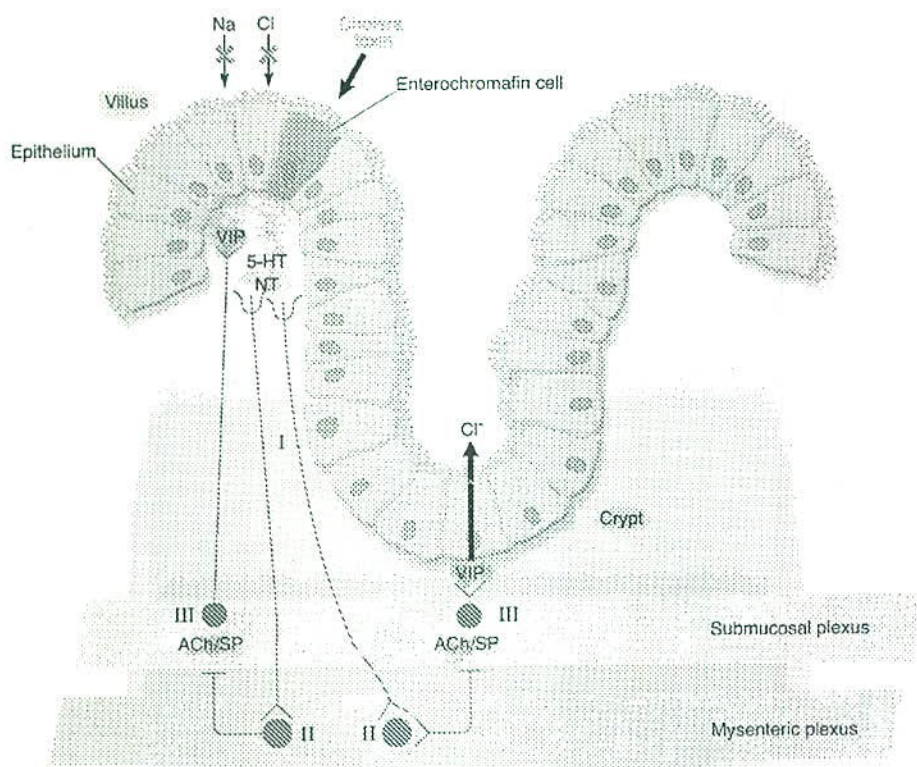


Figure 5

CT induces secretion, in part, through activation of an intramural neural reflex. Toxin attaches to enterochromaffin cells in the epithelium, causing an increase in [cAMP] there. In response to the latter, serotonin (5-HT), neurotensin (NT), and possibly additional peptides are released. These activate afferent neurons (I), whose axons course from close to the epithelium to the myenteric neural plexus, where they connect with interneurons (II) that, in turn, through release of acetylcholine (ACh) and/or substance P (SP), activate secretomotor neurons (III) in the submucosal neural plexus. Axons from these secretomotor neurons reach the epithelial surface, in both the villus and the crypt regions, releasing VIP and thereby stimulating secretion in crypts and inhibiting nutrient-independent salt absorption in villi. For a comprehensive review, see ref. 42.

disease, which includes severe villus atrophy and crypt hypoplasia with the characteristic finding of microvilli contained within vesicles inside the enterocytes.

Hormone-secreting neoplasms

In several uncommon tumors, hormones are produced and released that directly stimulate intestinal secretion, causing profuse diarrhea or, in one instance (gastrinoma), interfering with nutrient absorption. For a comprehensive review, see ref. 42.

Pancreatic cholera. In patients with pancreatic cholera, certain endocrine neoplasms that occur most commonly in pancreatic islets but occasionally in the proximal intestinal mucosa secrete large quantities of VIP, the enteric secretory neurotransmitter. Some ganglioneuromas (neural crest tumors seen in early childhood) and pheochromocytomas do so also.

Carcinoid syndrome. In carcinoid syndrome patients, cutaneous flushing and profuse diarrhea develop when carcinoid tumors in intestine metastasize to the liver, thereby not only amplifying the tumor mass but also bypassing hepatic detoxification of tumor-released hormones. Very rarely, bronchogenic carcinoids give rise to the syndrome. Carcinoids have been reported to secrete serotonin, bradykinin, substance P, and prostaglandins, all of which are secretory stimuli in the intestine.

Medullary carcinoma of the thyroid. Profuse diarrhea develops in 30% of patients with medullary carcinoma of the thyroid because of secretion of calcitonin, another secretory stimulus in the intestine.

Zollinger-Ellison syndrome (gastrinoma). In Zollinger-Ellison syndrome (gastrinoma), both diarrhea and pep-

tic ulceration can result from the marked increase in gastric acid production that is associated with gastrin-secreting neoplasms. The diarrhea, unlike those associated with the neoplasms just cited, is at least partly malabsorptive in nature. The marked increase in acid production both presents the intestines with a large volume for reabsorption and lowers pH in the proximal small bowel, inactivating pancreatic digestive enzymes and precipitating bile acids. Diarrheal stool in these patients has an acidic pH.

Systemic mastocytosis. About half of the patients with the rare neoplasm systemic mastocytosis develop diarrhea, likely due to histamine-induced gastric hypersecretion, a cause similar to that of the diarrhea in Zollinger-Ellison syndrome.

Ileal disease or resection

Nearly all conjugated bile acids are normally reabsorbed in the distal ileum via Na-bile acid cotransport, with very little reaching the colon (S76-S77). Small-bowel Crohn disease may lead to bile acid malabsorption, since distal ileum is frequently affected; also, obstruction there may develop, necessitating ileal resection. Unabsorbed dihydroxy bile acids such as deoxycholate are amphophiles that, when not incorporated into lipid-containing micelles in the gut lumen, tend to insert into the lipid matrix of the enterocyte or colonocyte brush border membrane. Deconjugation of these bile acids by colonic bacteria enhances their amphophilicity. They activate secretion primarily in the colon but, in the absence of lipids, can also do so in the small bowel. The mechanism for their secretory effect

is complex, involving both intracellular Ca^{2+} (probably secondary to membrane phospholipase activation) and cAMP (S78, S79). Bile acid-induced increase in cAMP appears to involve the ENS or mast cells (S80). Diarrhea that results from moderate malabsorption of bile acids can be somewhat reduced by cholestyramine, a resin that binds bile acid anions. But if more than 100 cm of distal ileum is resected, so much of the enteric bile acid load fails to be reabsorbed that the body's bile acid pool shrinks (43). Then, trapping of additional bile acids with cholestyramine shrinks the bile acid pool further, causing fat malabsorption and thereby worsening the diarrhea. This is because fatty acids that reach the colon undergo bacterial hydroxylation, which renders them amphophilic. Like amphophilic bile acids, and probably by a similar mechanism, they induce secretion (44).

Intestinal lymphectasia

Obstruction of intestinal lymphatics can be congenital or neoplastic. The resulting increase in interstitial hydrostatic pressure disrupts tight junctions, thereby greatly increasing epithelial permeability not only to water and electrolytes but also to serum proteins. Since chylomicrons are absorbed through lymphatics, fat ingestion by patients with intestinal lymphectasia will increase pressure in their partially obstructed intestinal lymphatics, thereby increasing enteric protein loss and diarrhea. These losses can be lessened by a low-fat diet supplemented with medium-chain triglycerides (which, being water soluble, are absorbed into the portal vein).

Tropical sprue and nontropical sprue (celiac disease, or gluten-sensitive enteropathy)

Sprue histopathology is characterized by total or subtotal villus atrophy, elongation of crypts with increased crypt mitoses, and a dense infiltrate of inflammatory cells, especially plasma cells. The surface epithelium reveals, in addition to flattening, a marked increase in intraepithelial lymphocytes and degenerative changes such as vacuolization and loss of brush border. There are four reasons for the profuse diarrhea that develops in some of these patients: (a) the osmotic force of unabsorbed solutes pulls water and electrolytes into the lumen; (b) villus atrophy and crypt hypertrophy adversely alter the balance between absorptive and secretory cells; (c) unabsorbed bile acids and fatty acids stimulate fluid secretion in the colon; and (d) the inflammatory response generates secretion-stimulating inflammatory mediators. $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$, whose concentrations in the lamina propria increase in celiac disease, also downregulate nutrient absorption (S73, S81–S83).

Diabetes mellitus

Diarrhea accompanied by rectal incontinence is an occasional complication of long-standing, insulin-dependent diabetes. It typically occurs in patients with poor diabetic control and peripheral neuropathy. Intestinal biopsies in such patients are usually normal, and nutrient malabsorption or bacterial overgrowth is present only in a minority of cases. In most instances,

the diarrhea is secondary to degeneration of adrenergic nerves that, as mentioned above, are antisecretory and/or proabsorptive in intestinal fluid homeostasis (45, S84). Other evidence of autonomic neuropathy can usually be elicited. There is anecdotal evidence that diabetic diarrhea can sometimes be controlled with an α_2 agonist such as clonidine or with a somatostatin analog such as octreotide (S85, S86).

Bacterial overgrowth

Normally, only a relatively small number of bacteria proliferate in the small bowel. In the presence of local stasis or recirculation of luminal contents, however, bacteria will proliferate in the affected segment of small bowel (e.g., afferent loop dysfunction after gastrojejunostomy; jejunal diverticula; strictures or enterocolic fistulas in Crohn disease; and ineffective peristalsis in scleroderma or diabetes). If the stasis occurs proximally, the bacteria will deconjugate and partially dehydroxylate bile acids, leading to their rapid diffusional reabsorption. The resulting decrease in concentration of effective bile salts can cause fat malabsorption. As mentioned above, lipids that are hydroxylated by bacteria are thereby converted into secretion-stimulating amphophiles. In addition, bacterial proteases can inactivate brush border oligosaccharidases, leading to carbohydrate malabsorption.

Inflammatory bowel disease

In regional enteritis (Crohn disease), damage to or resection of the distal ileum can lead to bile acid malabsorption, which, as outlined above, causes diarrhea. Also in Crohn disease, enterocolic fistulas may develop, resulting in bacterial overgrowth of the small bowel, and this, as outlined just above, causes diarrhea. In both ulcerative colitis and Crohn colitis, inflammatory mediators such as prostaglandins stimulate colonic secretion, contributing to diarrhea. Cytokines generated in the inflamed mucosa may also downregulate fluid-absorptive mechanisms (S73, S81–S83).

Principles of management

Oral rehydration therapy

Cholera and *E. coli* enterotoxins stimulate fluid secretion without injuring the intestinal epithelium; in particular, they do not impair the cellular processes for nutrient absorption. Even in viral enteritis, which does cause epithelial injury, considerable absorptive capacity remains. Long ago it was noted that, in cholera, diarrheal losses could not be replaced by oral administration of a simple salt solution — it only added to diarrheal volume. We now know why: the cAMP response to CT inhibits nutrient-independent salt absorption (i.e., Na/H and Cl/HCO_3 exchanges). In the 1960s, investigators in Dacca and Calcutta, mindful of the newly emerging concept of Na-coupled absorption of sugars and amino acids, found that, when they added glucose to an orally administered salt solution (an isosmotic solution with approximately equimolar amounts of Na^+ and glucose), cholera patients were

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able to absorb the solution (46, 47). In areas where the incidence of severe diarrhea is high and health workers and sterile intravenous solutions are scarce, the use of an oral rehydration solution administered by family members has saved many lives.

Although such solutions can replace diarrheal losses, they do not facilitate reabsorption of secreted fluid and therefore do not lessen diarrhea. Some reabsorption of secreted fluid can be achieved by substitution of glucose polymers for free glucose in the oral rehydration solution. Normally, the rate of hydrolysis of starch to maltose is slow compared with the rates of hydrolysis of maltose and of absorption of free glucose. Thus, as fast as glucose becomes available, it is absorbed, and hyperosmolarity of the luminal contents never develops. A glucose polymer can be thought of as a "glucose battery," providing a continuous supply of glucose for absorption under isosmotic conditions. Solutions containing extracts from rice or other grains are increasingly being used for oral rehydration and appear not only to replace fluid losses but also to lessen diarrhea (S87).

The same principle may apply to proteins and to the amino acids and oligopeptides generated from them, but there is relatively little experimental evidence of their efficacy.

Pharmacologic intervention

Antibiotics. To minimize development of drug-resistant organisms, antibiotics should not be used for relatively mild, self-limited infectious diarrheas. When the diarrhea is hemorrhagic, seriously dehydrating, associated with serious systemic signs and symptoms, or of longer than 5 days' duration without improvement, antibiotic use is appropriate. Fluoroquinolones are commonly used. *C. difficile*-induced diarrhea should be treated by withdrawal of the offending antibiotic and oral administration of either metronidazole or vancomycin.

Antidiarrheals. In mild diarrheas, antiperistalsis agents (e.g., loperamide) will lessen stool frequency and, by increasing the time of contact with the gut epithelium, will also lessen stool volume. Such agents are contraindicated in severe diarrhea, because they may cause pooling of large fluid volumes in paralyzed bowel loops.

Since intestinal ion transport mechanisms operate within a complex regulatory framework, drugs that either mimic or potentiate the effects of antisecretory/proabsorptive hormones or neurotransmitters are proving useful. Recently, an enkephalinase inhibitor, racecadotril (also known as acetorphan), has been shown to lessen the volume of acute infectious diarrhea in children, presumably by preventing breakdown of enkephalins in the mucosa, which are anti-secretory (S88, S89). Its action is confined to intestine and, unlike loperamide, it doesn't reduce transit time.

Somatostatin analogues (e.g., octreotide) will sometimes control diarrhea. In hormone-secreting neoplasms, they block hormone production by the tumor (42). Since they also appear to have a direct antisecretory effect on the gut epithelium (S90), they have been employed for treating cancer chemotherapy-induced diarrhea. Anecdotal experience with an α_2 agonist,

clonidine, suggests its utility in some diarrheas, especially in diabetic diarrhea, but severe side effects limit its usefulness; one side effect, postural hypotension, would be problematic in dehydrated individuals.

Thus far, drugs that block the actions of secretagogues, such as prostaglandin synthase inhibitors, have not proven effective as antidiarrheals perhaps because of the multiplicity of locally produced secretagogues.

Two cellular sites provide targets for the development of transport inhibitors: the apical anion channel (CFTR) and the basolateral cAMP-sensitive K^+ channel. In the laboratory, specific blockers for each have been identified. Of particular interest, a class of high potency inhibitors of the CFTR anion channel (thiazolidinones) has recently been identified through screening a large number of compounds in transfected cells (48). One of these, on a single intraperitoneal injection into mice (250 $\mu\text{g/kg}$), reduced CT-induced secretion by more than 90%.

Overview of treatment

Understandably, therapies for epidemic infectious diarrheas must differ somewhat from those for chronic diarrheas. Epidemic cholera is most effectively dealt with at the contamination level. Cholera vaccines were not covered in this review; they are only partially effective and then only temporarily. An effective oral vaccine is not available. Efforts to improve sanitation are of more practical value. In a recent study, simple filtration of drinking water through cloth, such as a flat fragment of an old sari, reduced cholera incidence in Bangladeshi villages by 50% (49). Once infection has occurred, ORS remains the mainstay of treatment.

Several drugs that either have proven or may prove useful in treating chronic and some acute diarrheas were cited above. In addition, diarrheas with an inflammatory component can be treated with anti-inflammatory agents, most notably glucocorticoids.

Note: Due to space constraints, a number of important references could not be included in this reference list. Interested readers can find a supplementary reference list at (<http://www.jci.org/cgi/content/full/111/7/931/DC1>).

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Duodenal biopsies of HIV-infected patients with diarrhoea exhibit epithelial barrier defects but no active secretion

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Objectives: To characterize diarrhoeal mechanisms in HIV-infected patients, epithelial transport and barrier function of the duodenal mucosa was investigated *in vitro*.

Patients: Twenty-one HIV-seropositive patients (13 asymptomatic and eight with diarrhoea) and 12 controls from an urban referral-based tertiary care centre in Berlin who underwent duodenoscopy.

Methods: A new miniaturized Ussing chamber allowed measurements on duodenal forceps biopsies. Epithelial barrier function was characterized by alternating current impedance analysis, which allows differentiation of epithelial and subepithelial resistance and by ^3H -lactulose and ^3H -mannitol flux measurements. Na^+ -glucose cotransport was quantified as phlorizin-sensitive short circuit current (I_{sc}) and active ion secretion by baseline and bumetanide-sensitive I_{sc} .

Results: Duodenal biopsies from asymptomatic HIV-infected patients were no different from controls, whereas biopsies from HIV-infected patients with diarrhoea showed a decrease in epithelial resistance from 21.2 ± 1.9 to $12.9 \pm 1.3 \Omega\text{cm}^2$ ($P < 0.01$). Concomitantly, mucosal-to-serosal lactulose flux increased from 0.29 ± 0.02 to $0.40 \pm 0.03 \mu\text{mol} (\text{hcm}^2)$ ($P < 0.01$). Phlorizin-sensitive I_{sc} indicating Na^+ -glucose cotransport, as well as baseline and bumetanide-sensitive I_{sc} indicating active electrogenic chloride secretion were not different between the three groups.

Conclusions: A miniaturized Ussing device was developed for electrophysiological investigations of duodenal forceps biopsies, which allowed characterization of active ion transport mechanisms and epithelial barrier function. Duodenum of HIV-infected patients with diarrhoea showed no evidence for active ion secretion or Na^+ -glucose malabsorption, but showed an impaired epithelial barrier function, which could contribute to diarrhoea by a leak flux mechanism.

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Keywords: HIV, AIDS, diarrhoeal mechanisms, duodenum, impedance, malabsorption, permeability, secretion, Na^+ -glucose cotransport

Introduction

Diarrhoea and malabsorption are frequent in HIV infection and important factors for mortality [1]. A potential enteric pathogen is detected in about two-

thirds of the patients with diarrhoea [2], whereas only in a minor percentage (15-40%) no infective agent is found and HIV itself may be important (HIV enteropathy) [3,4]. However, even if an enteropathogen is detected, the meaning of this can be uncertain because

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enteropathogens are also found in asymptomatic HIV-infected patients [5].

Several pathomechanisms have been assumed to contribute to diarrhoea. Fat malabsorption may be important and could be caused by lymphatic obstruction [6,7]. Furthermore, villus atrophy with and without crypt hyperplasia (i.e., hyper-regenerative mucosal transformation) has been found, but is often not consistent with clinical symptoms [8,9]. HIV has been detected in enterochromaffin cells [10] as well as in lamina propria mononuclear cells [7] or lymphocytes [8], which can lead to the release of inflammatory mediators. Levels of some cytokines are indeed increased in the intestinal mucosa in HIV-infected patients, as shown for tumour necrosis factor (TNF)- α , interleukin (IL)- 1β [11,12] and interferon (IFN)- γ [13]. Furthermore, previous studies have shown that TNF- α and IL- 1β can induce intestinal active Cl^- secretion [14,15]. However, it is not known whether active ion secretion is stimulated in HIV-infected patients.

Studies on intestinal cell lines have revealed that TNF- α and IFN- γ can, in addition to their transport effects, directly affect the epithelial barrier function by increasing tight junction permeability [16,17]. This could be important as *in vivo* permeability studies have detected abnormalities in symptomatic HIV-infected patients [18-21].

The aim of the present study was to characterize diarrhoeal mechanisms in HIV-infected patients by examining intestinal forceps biopsies using the Ussing technique. This technique is superior to other approaches such as *in vivo* perfusion or permeability studies due to its ability to distinguish active and passive transport processes and to relate permeabilities to a defined area reference. Thus, interference with factors such as variable intestinal transit time or small intestinal bacterial overgrowth is avoided [22,23]. For this purpose, we developed a miniaturized set-up that allowed us to measure on intestinal forceps biopsies. This enabled us to investigate epithelial barrier function and to quantify Na^+ -glucose cotransport and active ion secretion in the duodenum of HIV-infected patients using advanced electrophysiological techniques (alternating current impedance analysis) and conventional short-circuit current and flux measurements.

Materials and methods

Study subjects

HIV-seropositive patients (confirmed by enzyme-linked immunosorbent assay and Western blot) from an urban referral-based tertiary care centre in Berlin, who underwent endoscopy because of diarrhoea or other

symptoms, were included. Thirteen HIV-positive patients who had no history of diarrhoea represented the asymptomatic HIV group [Centers for Disease Control and Prevention (CDC) 1993 criteria stages A1-A2 or B1-B2]. Coronavirus was detected in three patients, *Giardia lamblia* in one patient, and *Clostridium difficile* in one patient. Only two patients received zidovudine, whereas the others were not taking any drug at the time of study. Eight HIV-positive patients with diarrhoea were included in the HIV diarrhoea group. Diarrhoea was defined as more than three loose or watery bowel movements per day for at least 1 week. All but one patient had AIDS-defining diseases according to the CDC criteria. Enteropathogens were detected in all patients: coronavirus in three patients; both cryptosporidia and coronavirus in two patients; a combination of microsporidia, coronavirus and cytomegalovirus in one patient; *Mycobacterium avium* complex (MAC) in one patient; and *Clostridium difficile* in one patient. All but one patient were treated with zidovudine, three in combination with zalcitabine, one in combination with didanosine, and one in combination with lamivudine. Twelve patients, who underwent upper endoscopy because of follow-up after *Helicobacter pylori* eradication or cancer search but had neither macroscopic nor histological abnormalities, served as controls. Clinical data of the patients are presented in Table 1. The study was approved by the local ethics committee and all patients gave written informed consent for taking extra biopsies for the study.

Investigations

Clinical history was taken at the time of the endoscopy, and included duration and frequency of diarrhoea. Screening for enteropathogens was performed using a standard protocol in small intestinal biopsies and stool samples [4,5]. Briefly, biopsies were taken from the duodenum for virological, microbiological and histopathological examination. In addition, three sets of faecal samples were examined for bacterial, protozoal, viral enteropathogens [24]. For the detection of microsporidia, stool was examined by trichrome stain and biopsy specimens by electron microscopy; cryp-

Table 1. Clinical data of study patients.

	Control	HIV asymptomatic	HIV diarrhoea
No. patients	12	13	8
Age (years)	48 (25-68)	39 (25-57)	39 (28-51)
Body mass index	25 (20-30)	24 (19-30)	20 (15-25)
CD 4 cell counts ($\times 10^6/l$)	-	449 (264-766)	78 (6-257)
Antiretroviral treatment (n)	0	2	7

Data are mean (range), unless otherwise indicated. Body mass index was calculated as weight (kg)/height (m)². HIV-infected patients with diarrhoea had lower body mass index than controls and asymptomatic HIV-infected patients ($P < 0.05$). HIV-infected patients with diarrhoea had lower CD 4 cell counts than asymptomatic HIV-infected patients ($P < 0.001$). CD 4 cell counts of controls were not measured.

tosporidia were detected by a modified cold Kinyoun acid-fast stain (Bio Mérieux, Marcy l'Etoile, France).

Miniaturized Ussing chamber

A miniaturized Ussing chamber with an opening diameter of 2.5 mm equivalent to an exposed area of 0.05 cm² was used (Fig. 1). All characteristics necessary for alternating current impedance analysis as well as conventional short-circuit current and flux measurements were provided in this design. The chamber preserved the advantages of conventional low volume gas lift stirred flux chambers [25]. Current electrodes were silver rings located far from the epithelium to form a homogeneous electrical field across the epithelium. The voltage electrodes were positioned axially within the electrical field. Voltage electrodes were covered by a driven shield and consisted of commercial microelectrode holders (MEH3SF, WP-Instruments, New Haven, Connecticut, USA) connected to glass micropipettes (not pulled; inner/outer diameter, 1.0/1.4 mm; length, 25 mm). The pipettes were filled with 3 g/dl purified agar in 0.5 mol/l KCl. Up to a frequency of 3 kHz, this Ussing chamber (including electrodes and amplifiers) exhibited no significant deviation from 'ideal' ohmic characteristics. Increasing but still very small inherent capacitance properties appeared above 3 kHz. At the highest frequency used, 65 kHz, the imaginary part of the complex impedance (Fig. 2) of the fluid-filled chamber (without tissue) was $-3 \Omega \text{cm}^2$. These deviations were corrected for in each single experiment (see below).

Experimental procedure

Biopsy specimens were obtained from the distal duodenum during upper endoscopy by a biopsy forceps with

an opening diameter of 3.4 mm. Under a dissection microscope, biopsy specimens were spread out and a perforated plastic disc was glued on the serosal side using Histoacryl tissue glue (B. Braun, Melsungen, Germany). This disc was inserted in a plastic container and mounted between the two halves of an Ussing chamber (Fig. 1). The time between taking the biopsy and mounting it into the Ussing chamber was about 30 min, during which time it was kept in oxygenated standard medium at 4°C. After an equilibration period of 20 min, impedance analysis, three 15 min flux periods, and blocking experiments with phlorizin and bumetanide were performed.

Alternating current impedance measurements

Impedance analysis was performed as described previously [26-29] using a programmable 1250 Frequency Response Analyzer in combination with a 1286 Electrochemical Interface (Solartron Schlumberger, Farnborough, Hampshire, UK). Briefly, sine-wave alternating currents ($35 \mu\text{A}/\text{cm}^2$ effective current) in the range of 1 Hz to 65 kHz were applied and the voltage responses detected. Each impedance measurement consisted of 48 measurements at increasing frequency and took about 1 min. The complex impedance was calculated on-line and stored on the hard disk of a personal computer. The impedance locus was plotted in a Nyquist diagram. Correction for the impedance of the experimental set-up was performed by measuring the impedance of the fluid-filled chamber and subtracting it for each frequency from subsequently measured impedance data. Correction was calculated by vector subtraction in the complex impedance plane. This procedure also included correction for bath resistance.

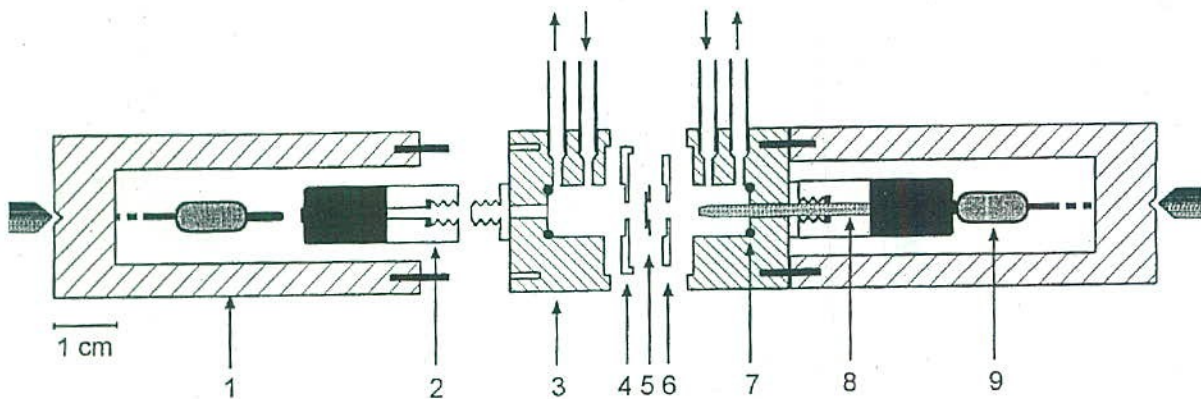


Fig. 1. Miniaturized Ussing chamber designed for electrophysiological investigations including alternating current impedance measurements of (duodenal) forceps biopsies. 1, Hollow cylinder transmitting force from the clamp pin of the vice and enclosing the microelectrode holder with a driven shield; 2, voltage electrode holder (WP-Instruments); 3, internal hemichamber with inlet and outlet for bubble-lift perfusion system (not shown) and a central bore for a glass pipette; 4, mucosal half of the milled plastic container; 5, forceps biopsy specimen glued onto a plastic disc; 6, serosal half of the plastic container; 7, current electrode (Ag/AgCl wire); 8, voltage electrode (microelectrode filled with 3 g/dl purified agar in 0.5 mol/l KCl); 9, connector and cable.

For each impedance locus plot, a circle segment was fitted to the data points by least squares analysis. From this circle segment three parameters of an electrical equivalent circuit were obtained, modelling the epithelium as a unit of a resistor (R^e) and a capacitor (C^e) in parallel and the subepithelium as a resistor (R^s) in series to this resistor-capacitor unit [30]. The inherent principle was that at the low frequency end of the impedance locus plot ($f \rightarrow 0$), the epithelial capacitor C^e was not conductive and the current had to pass R^e and R^s . Thus, intersection between the circle segment and the x-axis at the low frequency end represented total resistance (R^t). In contrast, at the high frequency end of the impedance locus plot ($f \rightarrow \infty$), C^e was completely conductive and bypassed the epithelial resistance (R^e). Thus, only the subepithelial resistance (R^s) remained (Fig. 2). Epithelial resistance (R^e) was then obtained as $R^t - R^s$. Further details of this method are given by Gitter *et al.* [26]. Thus, the impedance technique discriminated between the epithelial (R^e) and the subepithelial (R^s) contribution to the total wall resistance (R^t) without any mechanical preparation of subepithelial layers.

When total resistance (R^t) is altered, only impedance analysis can differentiate the extent to which R^e and R^s contribute to this change. Furthermore, the thickness of the subepithelium of a biopsy and thus of the respective R^s value could vary in pathologic conditions, as observed, for example, in one of our previous studies on the ileal mucosa in the J-pouch after colectomy [31]. Without knowledge of the epithelial/subepithelial resistance ratio, this can cause misinterpretation of measured barrier parameters and active transport rates [32].

Lactulose and mannitol fluxes

Short-circuit current (I_{sc}), total tissue resistance (R^t), and unidirectional 3H -lactulose and 3H -mannitol fluxes were measured using the Ussing technique [33] as described by Schultz and Zalusky [25]. For continuous registration of the electrical parameters, computer-controlled devices were used (Fiebig, Berlin, Germany). The standard medium contained Na^+ (140 mmol/l), Cl^- (123.8 mmol/l), K^+ (5.4 mmol/l), HPO_4^{2-} (2.4 mmol/l), $H_2PO_4^-$ (0.6 mmol/l), Ca^{2+} (1.2 mmol/l), Mg^{2+} (1.2 mmol/l), HCO_3^- (21 mmol/l), and as substrates, D(+)-glucose (100 mmol/l), β -OH-butyrate (0.5 mmol/l), glutamine (25 mmol/l) and D(+)-mannose (100 mmol/l). By gassing with 95% O_2 and 5% CO_2 , a pH of 7.4 at 37°C was obtained. For flux measurements, the medium also contained 10 mmol/l mannitol or 20 mmol/l lactulose. 3H -lactulose and 3H -mannitol (Biotrend, Cologne, Germany) were added to the mucosal side. Samples were taken from the mucosal and serosal side, and radioactivity was counted by an Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard, Meriden, Connecticut, USA). Fluxes (J) were calculated by the standard formula de-

scribed by Schultz and Zalusky [25]. The absence of a significant drift of the voltage electrodes was checked at the end of each experiment. All I_{sc} values were corrected for bath resistance, as described by Tai and Tai [32].

Phlorizin and bumetanide effects

After reaching steady state values for I_{sc} , 5×10^{-4} mol/l phlorizin (Sigma, St Louis, Missouri, USA) was added to the mucosal side. Subsequently, 10^{-5} mol/l bumetanide (Sigma) was given serosally.

Evaluation of miniaturized Ussing chamber on human duodenal biopsy specimens

Duodenal biopsy specimens from control patients were mounted into the miniaturized Ussing chamber (Fig. 1). After a equilibration period of 20 min, 10^{-2} mol/l theophylline (Sigma) was added on both sides, and 10^{-6} mol/l prostaglandin (PG)- E_2 (Boehringer Ingelheim, Heidelberg, Germany) was added on the serosal side. After an additional 20 min, 10^{-3} mol/l 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was added mucosally. NPPB (RBI, Natick, Massachusetts, USA) was dissolved in dimethylsulphoxide (DMSO). Maximum DMSO concentration in the chamber was 0.1%, a concentration which had no effect in control experiments.

Statistical analysis

All data are means $\pm$ SEM. Differences between groups were tested by analysis of variance and two-tailed Student's *t* test for unpaired data. SPSS for Windows software package (SPSS, Chicago, Illinois, USA) was used. A *P* value of < 0.05 was considered significant.

Results

Evaluation of miniaturized Ussing chamber on human duodenal biopsy specimens

Initially, total resistance (R^t) of duodenal biopsy specimens was $47 \pm 4 \Omega cm^2$ and I_{sc} was $121 \pm 14 \mu A/cm^2$ ($n = 8$). After simultaneous addition of the cAMP-dependent secretagogues theophylline and PGE_2 , I_{sc} increased to $255 \pm 34 \mu A/cm^2$ ($\Delta I_{sc} = 110\%$). Subsequently, after mucosal addition of the chloride channel inhibitor NPPB [34] I_{sc} was decreased again to $112 \pm 10 \mu A/cm^2$ after 40 min, resulting in complete inhibition of the theophylline/ PGE_2 -induced secretory response. Taken together, these data demonstrate that duodenal biopsy specimens in the miniaturized Ussing chamber were able to respond to secretory stimuli in a reliable manner. Thus, ongoing intestinal secretion in HIV infection, for example, due to increased cytokine levels, would have been detectable using this technique.

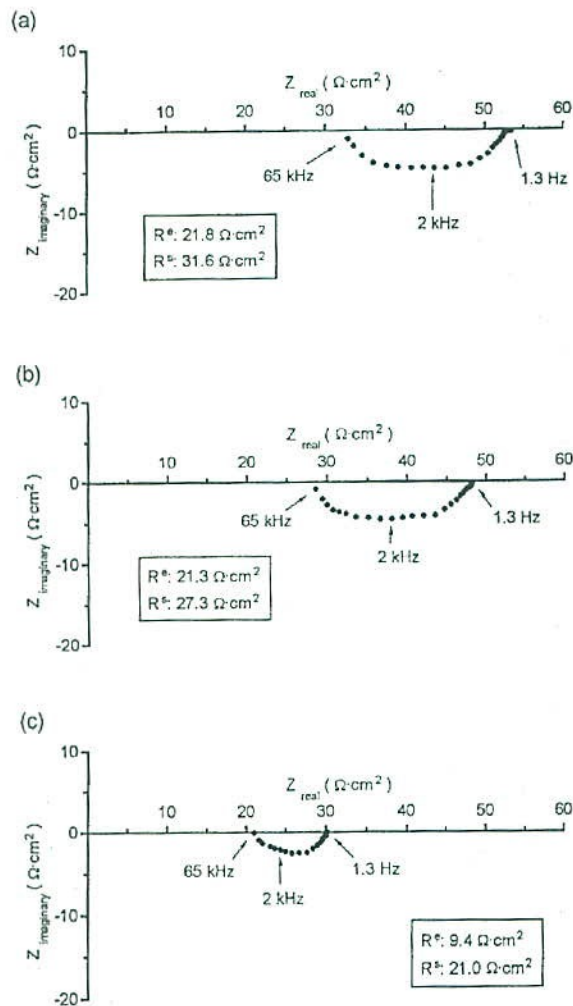


Fig. 2. Representative original impedance locus plots (Nyquist diagram) of duodenal forceps biopsy specimens from (a) a control patient, (b) an asymptomatic HIV-infected patient, and (c) an HIV-infected patient with diarrhoea. Z_{real} gives the ohmic component and $Z_{\text{imaginary}}$ the reactive component of the complex impedance. Intersections between the circle segment and the x-axis at low and high frequencies represent total resistance (R^t) and subepithelial resistance (R^s), respectively. Epithelial resistance (R^e) is $R^t - R^s$.

Alternating current impedance measurements

Original impedance locus plots of a control patient (Fig. 2a), an asymptomatic HIV-infected patient (Fig. 2b), and an HIV-infected patient with diarrhoea (Fig. 2c) are shown, and Table 2 shows the respective statistical analysis. Epithelial resistance (R^e) was $21.2 \pm 1.9 \Omega \text{ cm}^2$ in controls and was not significantly different in asymptomatic HIV patients ($19.5 \pm 1.8 \Omega \text{ cm}^2$), but was decreased to $12.9 \pm 1.3 \Omega \text{ cm}^2$ in HIV-infected patients with diarrhoea ($P < 0.01$). Subepithelial resistance (R^s) was $29.1 \pm 1.7 \Omega \text{ cm}^2$ in controls, unchanged

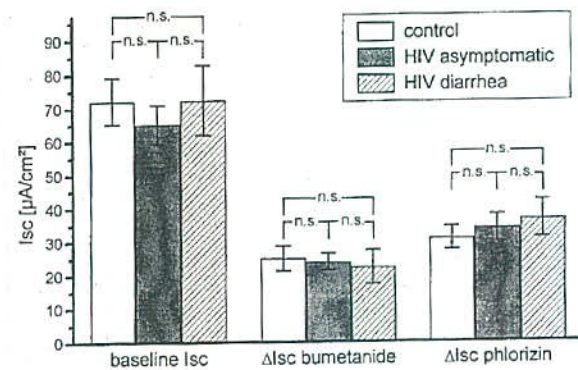


Fig. 3. Baseline short-circuit current (I_{sc}), bumetanide-sensitive I_{sc} and phlorizin-sensitive I_{sc} in HIV-infected patients and controls. All values are means $\pm$ SEM. NS, Not significantly different.

in asymptomatic HIV-infected patients ($30.4 \pm 1.4 \Omega \text{ cm}^2$), and only slightly diminished to $25.0 \pm 1.5 \Omega \text{ cm}^2$ in HIV-infected patients with diarrhoea. This difference was significant when compared with asymptomatic HIV-infected patients ($P < 0.05$), but failed to reach significance when compared with controls.

R^t/R^e is the factor by which measured transport rates (e.g., I_{sc} values) underestimate the true active transport. Whenever, in addition to the epithelial resistance, significant non-epithelial series resistances are present between the voltage-sensing electrodes, measured I_{sc} or net fluxes have to be corrected for the contribution of these resistances. This is well known for the bath resistance, but is also necessary for the subepithelial resistance of intestinal preparations. The implications of this correction are given elsewhere in detail [27–29,32]. R^t/R^e ratio was 2.4 ± 0.4 , 2.6 ± 0.4 and 3.0 ± 0.5 in the three groups, respectively. Since there was no significant difference between the three groups, we dispensed with correction of measured transport rates in this study. Thus, measured I_{sc} values underestimate the true active transport rates considerably, but to a similar degree in all three groups.

Lactulose and mannitol fluxes

The results of the flux experiments are shown in Table 2. No difference was found between asymptomatic HIV-infected patients and controls. In HIV-infected patients with diarrhoea, lactulose and mannitol fluxes were increased, although the increase in mannitol flux reached statistical significance only in comparison to the asymptomatic HIV-infected group. This was accompanied by a decrease in R^t . I_{sc} was not altered in HIV-infected patients.

Phlorizin and bumetanide effects

Phlorizin is a blocker of the Na^+ -glucose symporter in the apical enterocyte membrane. Thus, the decrease in

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Table 2. Impedance analysis, and lactulose and mannitol fluxes of duodenal biopsy specimens from control and HIV-infected patients either asymptomatic or with diarrhoea.

	Impedance analysis				Lactulose and mannitol fluxes				
	R ^e (Ωcm ²)	R ^s (Ωcm ²)	R ^t /R ^e	n	R ^t (Ωcm ²)	I _{sc} (μA/cm ²)	J _{Lactulose} [μmol/(hcm ²)]	J _{Mannitol} [μmol/(hcm ²)]	n
Control	21.2±1.9	29.1±1.7	2.4±0.4	10	47.0±2.3	91±8	0.29±0.02	0.15±0.02	10
HIV asymptomatic	19.5±1.8	30.4±1.4	2.6±0.4	10	49.9±2.0	87±7	0.29±0.01	0.11±0.01	12
HIV diarrhoea	12.9±1.3*†	25.0±1.5†	3.0±0.5	8	35.2±2.6*†	102±15	0.40±0.03*†	0.24±0.06†	8

All values are means ± SEM, unless otherwise indicated. Impedance analysis: R^e, Epithelial resistance; R^s, subepithelial resistance; R^t, total tissue resistance is the sum of R^e and R^s; R^t/R^e, correction factor of active transport rates for subepithelial resistance contribution. Flux experiments: R^t, total tissue resistance; I_{sc}, short-circuit current; J, mucosal to serosal lactulose and mannitol fluxes. *P < 0.01 versus control. †P < 0.05 versus asymptomatic HIV-infected patients. ‡P < 0.01 versus asymptomatic HIV-infected patients.

I_{sc} after addition of phlorizin is a measure of Na⁺-glucose cotransport. There was no significant difference in the decrease of I_{sc} (ΔI_{sc}) in response to mucosal addition of phlorizin in HIV-infected patients either without or with diarrhoea when compared with the controls (Fig. 3). ΔI_{sc} was 31 ± 4 μA/cm² in controls (n = 11), 34 ± 4 μA/cm² in asymptomatic HIV-infected patients (n = 13), and 37 ± 6 μA/cm² in HIV-infected patients with diarrhoea (n = 8).

The baseline I_{sc} remaining after addition of phlorizin is a measure of active electrogenic transport including electrogenic chloride and bicarbonate secretion. It was not significantly different between the three groups (Fig. 3). Baseline I_{sc} was 72 ± 7 μA/cm² in controls (n = 11), 65 ± 6 μA/cm² in asymptomatic HIV-infected patients (n = 13), and 72 ± 10 μA/cm² in HIV-infected patients with diarrhoea (n = 8). Thus, there was no evidence for activation of an electrogenic secretory system in the duodenum.

In addition, bumetanide-dependent ΔI_{sc} was not significantly different (Fig. 3). The decrease in I_{sc} after addition of bumetanide was 25 ± 4 μA/cm² in controls (n = 11), 24 ± 3 μA/cm² in asymptomatic HIV-infected patients (n = 11), and 22 ± 5 μA/cm² in HIV-infected patients with diarrhoea (n = 7). Bumetanide is a blocker of the basolateral Na⁺-2Cl⁻-K⁺ symporter of the enterocytes, which is part of the active chloride secretory system. Thus, there was no evidence for activation of active anion secretion in HIV-infected patients.

Discussion

A wide range of diarrhoeal mechanisms has been postulated in HIV infection. In general, according to the underlying mechanisms, diarrhoeal states can be divided into four subgroups [35]: (i) motility-dependent diarrhoea (not further considered in the present study); (ii) malabsorptive/maldigestive diarrhoea, where ingesta are not taken up as a consequence of impaired absorption or reduced enzymatic digestion, and subsequently,

equivalent amounts of water are osmotically retained in the intestinal lumen causing diarrhoea; (iii) secretory diarrhoea, where active electrogenic chloride or bicarbonate secretion is activated, for example, caused by enterotoxins; (iv) leak flux-induced diarrhoea, in which epithelial barrier impairment can induce a passive leak flux of ions, substrates, and water, as a consequence of which diarrhoea takes place (the experimental evidence for this diarrhoeal mechanism is further discussed below).

With the technique presented here, a direct characterization of epithelial transport and barrier properties in duodenal biopsy specimens of HIV-infected patients was possible *in vitro* for the first time. In particular, we were able to analyse the induction of active intestinal ion secretion as well as barrier function with defined area reference in a defined intestinal segment.

In this study, a 40% decrease of epithelial resistance was observed in HIV-infected patients with diarrhoea indicating an impaired epithelial barrier function. This means that a leak flux mechanism could be a possible cause for the diarrhoea. Such a passive leak flux of ions and water is best characterized in the case of *Vibrio cholerae* infection [36], where a second toxin, zonula occludens toxin (ZOT), was detected in a vaccination strain of cholera. This strain was genetically depleted of the cholera toxin, but still caused significant watery diarrhoea. As directly shown for ZOT in *V. cholera* infection, downregulation of tight junction permeability could also be induced in HIV infection. In the case of HIV, this could be mediated by a release of TNF-α or IFN-γ, since these cytokines can influence tight junction permeability [16, 17] and are elevated in the intestinal mucosa in AIDS [11-13].

In asymptomatic HIV-infected patients, neither epithelial nor subepithelial resistance was altered in our study. Additional factors have to be assumed to explain the alteration in advanced HIV infection. On the one hand, this could be due to (various) enteropathogens being pathogenic or even just present only in advanced stages of HIV infection. These enteropathogens could either act directly or via elevated cytokine levels. On the other hand, other factors could also be involved.

Batman *et al.* [37] proposed an autoimmune reaction of lamina propria immune cells against the patient's own HIV-infected cells. In this graft-versus-host phenomenon, cytokine release or activated cytotoxic T cells could also be important. The time in the course of HIV infection at which this mechanism may become apparent would then depend mainly on the viral dynamics as well as on immune parameters of the host [38].

Lactulose and mannitol fluxes were also found to be altered in symptomatic but not in asymptomatic HIV-infected patients, supporting the result of our impedance analysis. With the exception of one *in vivo* permeability study in which an abnormal permeability was found in early HIV infection [20], this corresponds to the finding of several *in vivo* permeability studies that an abnormal permeability is only observed in advanced HIV infection [18,19,21]. In these *in vivo* permeability studies, lactulose urinary recovery rate was found to be increased, whereas monosaccharide uptake was unchanged or even decreased. In contrast, in our *in vitro* measurements, not only lactulose but also mannitol flux was increased. This discrepancy is most likely due to different experimental conditions, as already noted by Krugliak and coworkers [39]. There is no carrier-mediated transport of mannitol and it has been shown that the majority of mannitol traverses the intestinal epithelium paracellularly [39-41]. Thus, a significant proportion of mannitol is assumed to be taken up by solvent drag, and decreased net water absorption in patients with diarrhoea could influence the mannitol uptake *in vivo* [39]. In contrast, lactulose as a disaccharide is too large to pass the normal tight junction and is therefore unlikely to be influenced by this factor [42].

Taken together, our *in vitro* study with defined area reference has identified for the first time a barrier impairment for tracers of either size, namely lactulose, mannitol, and small ions (by impedance analysis). As far as ion conductance is concerned, this is the first piece of evidence for an increased permeability in a group of the HIV-infected patients with diarrhoea. In contrast to disaccharide or macromolecule permeability, a change in ion permeability can cause diarrhoea by a leak flux mechanism.

There was no detectable Na^+ -glucose malabsorption in the duodenum of HIV-infected patients in our study. On the first view, this seems to differ from other studies that have found impaired glucose uptake [18-20]. However, these studies measured the urinary recovery rates of 3-*o*-methyl-D-glucose or D-xylose, but did not test the Na^+ -glucose cotransport system directly on the cellular level. The fact that the Ussing technique is indeed able to detect glucose malabsorption is evident from our previous study in the blind-loop syndrome [29]. We concluded from our data that there was no

impairment of glucose absorption, at least on the level of the enterocyte brush border membrane, which is characterized by the phlorizin-sensitive decrease in I_{SC} . Thus, other factors, such as small intestinal bacterial overgrowth or motility dysfunction, exist to explain the findings of glucose malabsorption in *in vivo* permeability studies [22,23].

This is the first study to characterize active ion secretion in HIV-infected patients with diarrhoea using the Ussing technique. We found no evidence for activation of active ion secretion. Neither baseline I_{SC} nor bumetanide-sensitive I_{SC} were altered. This is direct experimental evidence against activation of active ion secretion, since the two most important anion secretory systems, chloride and bicarbonate secretion, would have been detectable by these electrical parameters.

In principle, active ion secretion could be activated by different enteropathogens. Therefore, our results in HIV-infected patients with diarrhoea only point against a secretory effect of the HIV infection itself, but cannot rule out that active ion secretion could be important in a single patient with a secondary enteropathogen. To detect this, further subclassification for enteropathogens would have been required. However, even a study dealing with a single enteropathogen, namely cryptosporidia, for which activation of active secretion was observed in an animal model [43], failed to detect active ion secretion in humans [44]. Here, no evidence for an shift of ion and water transport towards secretion was observed in AIDS patients with diarrhoea with a jejunal perfusion technique [44]. Taken together, our results point strongly against a secretory type of diarrhoea in HIV-infected patients by HIV itself.

This can also explain the findings of a multicentre trial of octreotide therapy in AIDS patients with diarrhoea. In this study, octreotide therapy was not effective in reducing diarrhoea in the majority of patients [45]. Bearing our results in mind this is not surprising if diarrhoea in most HIV-infected patients is not of a secretory type. Thus, only few patients with enterotoxin-producing enteropathogens may profit from an octreotide therapy.

In conclusion, the impaired epithelial barrier observed in our study suggests a leak flux mechanism as an important cause of diarrhoea in HIV-infected patients. The interpretation of this mechanism is underlined by our findings that there is no activation of active secretory processes such as electrogenic Cl^- secretion and no evidence for Na^+ -glucose malabsorption at the enterocyte level in the duodenum. This does not encourage attempts for antisecretory therapy and should turn our attention to the epithelial barrier function of the intestine. However, before respective therapies can be considered, the inherent pathophysiological mechanisms

have to be elucidated. Possible candidates in regulating the epithelial barrier are cytokines such as TNF- α and IFN- γ .

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Pilot Study of Sandostatin (Octreotide) Therapy of Refractory HIV-Associated Diarrhea

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Seventeen AIDS patients were enrolled in a prospective open-label dose-finding study of octreotide (Sandostatin) therapy for refractory diarrhea. Five were nonevaluable due to progression of AIDS symptomatology, and one was excluded because of lack of confirmation of HIV infection. Five of 11 evaluable patients responded to therapy (45%); two each at 50 µg and 100 µg, and one at 250 µg thrice daily doses. A sixth patient demonstrated a moderate reduction in stool volume at 250 µg thrice daily, which, although deemed clinically relevant, did not meet the criteria for response. On discontinuation of therapy, diarrhea recurred in all patients within 1-12 days, and responded to reinitiation of octreotide in those five patients who resumed treatment. Only one of the three patients with concurrent cryptosporidial infection responded to treatment. The drug was well tolerated, with mild symptomatology in three patients. Long-term treatment at a stable dose was effective in three of five treated patients for periods for seven months in one (moderate responder) and one year in two. One patient required dose increases to control symptoms, but after one year of treatment developed severe nausea following injections, which required dose cessation. One patient had partial control of his diarrhea for only three months despite two dose increases. These data suggest that octreotide may be of useful therapeutic value in HIV-associated diarrhea and that further studies are indicated.

KEY WORDS: octreotide; Sandostatin; HIV; diarrhea; therapy.

Gastrointestinal disorders are common in association with HIV infection. Although one or more enteric pathogens can be isolated from up to 85% of HIV-

infected patients, many of these are poorly responsive to treatment with specific anti-infectious drugs (1-7). In addition, a noninfectious enteropathy characterized by malabsorption and mucosal abnormalities of the small intestine has been infrequently observed (8, 9), presenting with profuse watery diarrhea, which is often refractory to standard antidiarrheal therapies such as loperamide, opium tincture, and diphenoxylate. Octreotide (Sandostatin), a long-acting somatostatin analog (10), has been shown to be effective in the therapy of secretory diarrheas due to VIPomas and carcinoid tumors (11). Anecdotal reports suggest that it may be useful in reducing stool output in

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patients with AIDS, especially in association with cryptosporidial infection (12-14). Thus, we undertook a pilot study to evaluate the safety and efficacy of octreotide in the treatment of AIDS-associated diarrhea and to determine the therapeutic dose range.

MATERIALS AND METHODS

Between November 1987 and March 1989, all eligible patients were identified from the practices of the investigators. Not all eligible patients were enrolled due to patient or physician concerns regarding the therapy, mode of administration, or severity or illness of the patient. Seventeen patients were enrolled in this prospective open-label multicenter study. The study design was a dose-escalating trial. Patient eligibility was defined as follows: (1) documented HIV infection by confirmatory antibody testing, (2) clinical features of AIDS or of HIV infection defined by the CDC classification (15, 16), and (3) diarrhea unresponsive to at least one week of conventional antidiarrhea therapy. All enrolled patients had had more than one month of diarrhea prior to their enrollment in the study. All patients had stool testing done for bacterial and parasitic pathogens, *Cryptosporidium* and *C. difficile*. If enteric pathogens were isolated, the infection had to be refractory to previously administered specific therapy. Diarrhea was defined as more than three unformed bowel movements per day and/or approximately 1 liter/day of stool volume. Rectal exams were not performed routinely. However, monitoring of symptoms of tenesmus as an indicator of rectal disease was performed throughout the study.

Octreotide (Sandostatine) Sandoz Pharma Ltd., Basel, Switzerland) treatment was initiated at 50 µg thrice daily subcutaneously and the dosage escalated every 48 hr if diarrhea was not controlled at each of the successive dosage levels (50 µg, 100 µg, 250 µg, 500 µg thrice daily). If diarrhea was not controlled after one week at the maximal dosage (500 µg thrice daily), the medication was withdrawn. If diarrhea was controlled at one of the four dosage levels, the patient was maintained on that efficacious dose for two weeks. Once diarrhea had been controlled for two weeks, octreotide therapy was discontinued to evaluate whether diarrhea recurred upon drug withdrawal. If diarrhea recurred, reinstitution of therapy was planned at the last efficacious dose, 48 hr after its recurrence. Patients who resumed therapy were monitored by their physicians under a compassionate protocol with monthly clinical and laboratory evaluations.

Most patients were admitted to hospital for the duration of the study. Individuals were evaluated for two days prior to treatment and daily during the course of octreotide administration. Diarrhea was documented by the use of a daily stool diary, completed by the patient and/or care-giver, itemizing number and consistency of bowel movements. Stool was collected in containers and the 24-hr volume was determined. Complete laboratory evaluations including hematology, biochemistry, and urinalysis were done at regular intervals during the study. Adverse event monitoring was carried out.

Efficacy was defined as a decrease in the number of stools to equal to or less than three per day and/or 50% decrease in daily stool volume and maintenance of this response for a two-week treatment period. Ethics approval was obtained for the study at each study site. Statistical evaluation was carried out by means of the Wilcoxon signed ranks test for paired data.

RESULTS

Seventeen patients were enrolled in the study. One patient was withdrawn from data analysis because the diagnosis of HIV infection could not be confirmed on follow-up testing. Five subjects were nonevaluable due to progression of AIDS during the course of the study. Four of these patients died between study day 4 and 8 and the fifth experienced neurologic deterioration, which made it difficult to quantify his gastrointestinal symptoms.

Eleven patients completed the trial: two women and nine men with a mean age of 38 ± 11 (range 24-65). Three individuals were CDC stage IVA with diarrhea for greater than one month and weight loss of >10% body weight. Three had an initial AIDS diagnosis of *Pneumocystis carinii* pneumonia, and in four cases, the intestinal pathogens *Isospora belli* and *Cryptosporidium* constituted their AIDS-defined diagnosis. Only one patient had an active opportunistic infection during the course of the study (patient 8, toxoplasmosis). The duration of AIDS varied from one month to 16 months (mean 5.1 ± 4.5). The patients had mean Karnofsky scores of 58 ± 20 (range 30-90). Fifty-five per cent of the patients had previously documented intestinal pathogens, but at the time of drug administration only three patients had pathogens isolated, i.e., *Cryptosporidium* (patients 4, 6, and 14).

Pretreatment intestinal symptoms are presented in Table 1. All patients had increased frequency of bowel movements with a mean number of 8 ± 4 per day (range 3-14) associated with a marked weight loss over the previous three months. Stool volume was only measured in 6 subjects but ranged from 1.0 to 4.4 liters/day in these individuals.

Five of the 11 evaluable patients (45%), responded to treatment with octreotide (Table 1). The total daily dose leading to a therapeutic response was as low as 50 µg thrice daily in two patients, 100 µg thrice daily in two, and 250 µg thrice daily in the remaining patient. Of the three individuals harboring *Cryptosporidium* infection at the time of the study, only one had a good response to therapy.

TABLE 1. PRE-TREATMENT SYMPTOMATOLOGY AND RESPONSE TO THERAPY

Patient	Duration of diarrhea (mo)	Weight loss over previous 3 months (kg)	Mean bowel movements/day	Mean stool volume/day	Response/maximal dose†
1	3	16	11	2515	Good response, 100 µg TID
2	2	8	6	4428	No response, 500 µg TID
3	6	9	3	1375	Mod. response, 250 µg TID
4*	3	12	5	1329	No response, 500 µg TID
5	3	6	8	1550	No response, 500 µg TID
6	10	6	12	ND†	Good response, 50 µg TID
7	1	35	13	ND	No response, 500 µg TID
8	1	7	5	ND	Good response, 50 µg TID
9*	7	3	13	ND	No response, 500 µg TID
10	4	17	7	ND	Good response, 100 µg TID
11	16	8	5	984	Good response, 250 µg TID

**Cryptosporidium* isolated at time of therapy.
†ND = not done due to incontinence > 3 times/day.
‡Overall response (5/11)=45%; dose resulting in response: 50 µg TID (2), 100 µg TID (2), 250 µg TID (1); mod. = moderate response: 250 µg TID (1).

The effect of octreotide therapy on the frequency of bowel movements among responders is presented in Table 2. To compare baseline, treatment, and drug withdrawal periods, the mean number of bowel movements per day per patient were calculated for the baseline (2–4 days), maintenance (9–16 days), and drug discontinuation periods (2–7 days). The Wilcoxon-signed ranks test for paired data showed the stool numbers to be significantly reduced during the maintenance period as compared to baseline ($p = 0.0431$) and increased compared to the maintenance period following drug discontinuation ($p = 0.0431$). Stool volume also was reduced markedly in the patient with complete measurements. Recurrence of diarrhea occurred at a mean interval of 5.2 ± 4.5 days (range 1–12 days) off the drug.

A sixth patient demonstrated a moderate reduction (35%; 1375 ml to 900 ml) in stool volumes at a dose of Sandostatin of 250 µg thrice daily. This reduction,

while not meeting the efficacy criteria defined in the study protocol, was felt to be clinically relevant by the investigator in terms of the patient's nutritional status and quality of life. When Sandostatin therapy was withdrawn from this patient, stool volumes increased to 178% (1600 ml) of on-treatment values within four days.

All but one patient, who died shortly after the study, were restarted on their previous efficacious dose of octreotide and diarrhea was once again controlled. These five patients have been maintained on long-term octreotide. Three have been stable on the same dose: one for seven months, when he died (moderate response), and two continue at present for more than one year. One patient required a dose increase after six and then seven months to a maximum of 500 µg thrice daily, which stabilized his symptoms until he discontinued the drug after one year due to side effects. Another maintained partial control of his diarrhea for three months despite two dose increases to a maximum of 500 µg thrice daily. He discontinued treatment because of perceived lack of efficacy.

Tenesmus was recorded at varying times throughout the study in four of the 11 evaluable patients. In no patient was tenesmus present at all visits. In the responding patients, tenesmus was present inconsistently in patients 1, 10, and 11, and the nonresponding patient 2 reported tenesmus only at initiation and end of the study. However, the large volume of stool output experienced by these patients suggests that the diarrhea was not related to rectal disease.

Toxicity related to therapy with octreotide was minimal. Mild symptoms associated with drug ad-

TABLE 2. EFFECT OF OCTREOTIDE ON NUMBER OF DAILY BOWEL MOVEMENTS

Mean Number of Bowel Movements/day* (Stool volume, ml)			
Patient	Baseline period	Maintenance period	Drug discontinuation
1	11 (2515)	3 (700)	19 (4650)
6	12 (ND†)	2 (ND)	6 (ND)
8	5 (ND)	1 (ND)	7 (ND)
10	7 (837)	3 (ND)	10 (ND)
11	5 (984)	3 (ND)	6 (ND)

*Wilcoxon signed ranks test (two-tailed); $p = 0.0431$, baseline vs. maintenance, $p = 0.0431$, maintenance vs. drug discontinuation periods.
†ND = not done.

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ministration occurred in two: one had abdominal pain, one had nausea following injection and pain at the site of injection. One additional patient experienced severe nausea immediately after each injection, which began only after one year of treatment and led to discontinuation of the drug. Three patients developed abnormalities in liver function tests while on treatment. The relationship of these abnormalities to therapy is unclear. Two patients had minimal elevations of SGOT and alkaline phosphatase, respectively, and one showed a further increase in already abnormal SGOT, alkaline phosphatase, and triglycerides. Six patients reported no adverse side effects associated with therapy.

DISCUSSION

The management of an HIV-infected patient with diarrhea refractory to conventional therapy remains difficult. This group includes patients without documented pathogens as well as those who have cryptosporidial or other intestinal infections. This syndrome can be associated with marked weight loss, dehydration, electrolyte abnormalities, and severe debility (8, 9). Previous case reports have suggested that octreotide (Sandostatin) might be efficacious in reducing stool output in these patients, particularly when cryptosporidial infection is present (12-14). In addition, octreotide has been demonstrated to be efficacious at the dosage levels reported here in 57% (4/7) of AIDS patients treated on a compassionate need basis (unpublished data).

In this study, octreotide in doses ranging from 50 to 250 µg thrice daily was found to be effective in reducing the number of daily bowel movements and/or stool output in 45% of the treated patients. Despite early case reports suggesting that this drug may be useful in cryptosporidial infection (12-14), only one of the three patients with concurrent infection responded to treatment. The drug was well tolerated by the patients, with only one individual withdrawing from therapy because of severe nausea associated with injections, first noted after receiving therapy for a full year. Long-term therapy at a stable dose was successful in three of the five treated patients for more than a year in two and seven months in one. Two individuals required progressive dose increase. One maintained a good response for a year, when the drug was discontinued because of side effects. One had partial control for only three months.

The somatostatin analog octreotide has been shown to be an effective therapeutic agent in the treatment of hypersecretory disorders such as VIPomas and carcinoid tumors (11). The native hormone somatostatin stimulates salt and water absorption (11), decreases stimulated chloride secretion (17, 18), and inhibits the release of several gut hormones that contribute to diarrhea (18). Octreotide is a synthetic analog of somatostatin with similar biological activity but a prolonged half-life and thus overcomes the limitations of treatment with the native hormone.

The mechanism of action of octreotide in HIV-associated diarrhea is not fully understood. However, its effect on absorption, motility, and gut hormones may contribute to the observed therapeutic effect in these patients. Diarrheal syndromes observed in association with HIV infection may be heterogeneous. The patients reported here were characterized by the presence or absence of untreatable pathogens on routine stool tests, but endoscopic exam was not routinely carried out, fecal fat was not measured, and mycobacterial, cytomegalovirus, and microsporidial infections were not diagnosed, if present. However, the results obtained in this study suggest that the drug may be of therapeutic value in this group of patients. Further studies are indicated to confirm these observations and to define subgroups in which efficacy may be greater.

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Effect of long-term treatment with octreotide on rectal sensitivity in patients with non-constipated irritable bowel syndrome

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SUMMARY

Background

Acute administration of octreotide reduces visceral perception and therefore has been suggested as potential treatment for irritable bowel syndrome. Whether prolonged treatment with octreotide also reduces visceral sensitivity and improves gastrointestinal symptoms remains, however, unknown.

Aim

To investigate the effect of a slow release preparation of octreotide on rectal sensitivity and symptoms in irritable bowel syndrome patients.

Methods

Forty-six non-constipated irritable bowel syndrome patients (52% female, 19–63 years) participated. Before and after 8 weeks of treatment with octreotide (Sandostatin LAR 20 mg i.m.) or placebo, patients underwent a barostat study to assess the rectal sensitivity. During a 2-week run-in period and treatment, abdominal pain, defecation frequency, consistency and symptom relief were scored weekly.

Results

Octreotide, but not placebo, significantly increased the threshold for first sensation. Thresholds for urge to defecate and discomfort/pain and rectal compliance were not altered by either treatment. Octreotide improved stool consistency compared with placebo (loose stools after eight weeks: octreotide: 52%, placebo: 81%, $P < 0.05$). In contrast, abdominal pain and defecation frequency were not affected.

Conclusions

Although the threshold of first rectal sensation increased and stool consistency improved, long-term treatment with octreotide, at least at the current dose used, has no visceral analgesic effect and fails to improve irritable bowel syndrome symptoms.

Aliment Pharmacol Ther 26, 605–615

INTRODUCTION

The irritable bowel syndrome (IBS) is the most common disorder seen in the gastroenterological outpatient practice, accounting for approximately 28% of all diagnoses.¹ IBS is diagnosed on the basis of a series of clinical symptoms in the absence of organic diseases. These symptoms consist of chronic abdominal pain or discomfort, associated with altered defecation and changes in bowel habit. Symptom subgroups of IBS can be distinguished based on the predominant bowel habit, i.e. constipation predominant IBS (IBS-C), diarrhoea predominant IBS (IBS-D) and IBS with alternating constipation and diarrhoea (IBS-A). Several studies have shown that IBS patients exhibit enhanced perceptual responses to controlled distension of the colon and rectosigmoid.²⁻⁵ This so-called visceral hypersensitivity is a consistent finding in IBS and widely accepted as an important pathophysiological mechanism in generating IBS symptoms.⁶⁻⁹ Agents normalizing visceral sensation theoretically are potential new treatments for IBS.

One possible candidate drug is the somatostatin analogue octreotide. In acute experiments, administration of octreotide reduces perception of rectal and gastric distension in healthy volunteers¹⁰⁻¹³ and the perception of colonic and rectal distension in IBS patients.¹⁴⁻¹⁶ As such, octreotide may be useful to normalize hypersensitivity in IBS patients. Especially, as water and electrolyte absorption are increased and secretory diarrhoea is reduced by octreotide,¹⁷⁻²⁰ octreotide may be an interesting drug to treat diarrhoea predominant and alternating type IBS. An early case report indeed suggests that long-term treatment with octreotide can provide relief in IBS symptoms.²¹ However, at present, no controlled data are available from long-term treatment of IBS with somatostatin analogues. Therefore, we evaluated the effect of Sandostatin LAR, a slow release preparation of octreotide used in the treatment of acromegaly on rectal visceral sensitivity and IBS symptoms in non-constipated IBS patients.

PATIENTS AND METHODS

Study subjects

Forty-six non-constipated IBS patients (52% female, mean age 38 ± 2 years, range: 19–63 years) were recruited between September 2002 and November 2004 from the gastrointestinal motility unit at the

Academical Medical Centre, a tertiary referral centre. Selection criteria included a positive diagnosis by Rome II criteria of diarrhoea predominant or alternating type of IBS,²² excluding those with constipation predominant IBS. A minimum work-up to exclude organic disease included careful history taken, a normal physical examination, normal thyroid-stimulating hormone levels and blood counts, negative stool examinations and a normal flexible sigmoidoscopy. Biopsies of normal colonic tissue were routinely taken to exclude microscopic colitis. Patients had to be free of concomitant diseases (e.g. diabetes and cardiovascular disorders), including psychiatric disorders. Medication likely to interfere with gastrointestinal function or analgesics was discontinued at least 7 days before entering the study. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Centre. All participants gave their written and informed consent to participate in the study. Basal rectal sensitivity in IBS patients is compared with a matched historical control group that underwent the same barostat protocol.³ This historical control group consists of 17 healthy volunteers (47% female, mean age 39 ± 4 years) and were recruited by public advertisement.

Study drug

Sandostatin LAR (Novartis Pharmaceuticals Corp., East Hanover, NJ, USA) is a microencapsulated octreotide acetate suspension and is prepared immediately before administration. A vial containing the powder with Sandostatin LAR is gently wetted with 5-mL diluent and moderately swirled for 30–60 s until a milky, uniform suspension is achieved. A 19-gauge needle is used to inject this suspension with a steady pressure intra-muscularly in the gluteal region. Serum octreotide levels rise from the end of the first week to a steady concentration, which is maintained to approximately 5-weeks postinjection.¹⁸

Study design

This study is a placebo-controlled, double-blind, randomized trial, consisting of a 2-week screening period followed by an 8-week treatment period. Patients were randomized 1:1 for either treatment with intra-muscular injections of octreotide 20 mg or placebo. Before and after the treatment period, a rectal barostat study was performed to assess rectal sensitivity and IBS-symptoms were scored every week.

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Barostat studies

To assess the sensitivity to rectal distension, we used an electronic barostat that automatically corrected for the compressibility of air (Synetics Visceral Stimulator, Stockholm, Sweden) (Figure 1). The barostat was connected to a 500-ml polyethylene bag (maximal diameter 9 cm), tightly wrapped on the distal end of a double lumen polyvinyl tube (Salem Sump tube 14 Ch.; Sherwood Medical, St Louis, MO, USA) that was introduced in the rectum.^{3, 23}

Minimal distending pressure (MDP) was defined as the minimum pressure at which the intrabag volume was >30 mL. Subjects scored the perception of sensation evoked by rectal distension using a 6-point scale with verbal descriptors (0 = no sensation; 1 = first sensation; 2 = first urge to defecate; 3 = normal urge to defecate; 4 = severe urge to defecate; 5 = discomfort/pain). If a subject reported discomfort or pain, the bag was instantaneously deflated. For safety, the bag was automatically deflated at pressures above 50 mmHg or volumes above 600 mL.

Phasic distensions

The first rectal distension series was performed according to a phasic, semi-randomly ascending isobaric distension protocol. The pressure increment was 3 mmHg above MDP (3, 6, 12, 9, 18, 15, 24, 21, 30 mmHg, etc.), each step lasting 2 min and separated by 1-min intervals at baseline (MDP). The inflation rate was set at 38 mL/s. Sensations were scored after 10, 60 and 150 s of each distension step. Sensation was determined as the highest score during each distension step, independently of timing.

Volume ramp distension

Subsequently, after a 15-min recuperation period with the bag deflated, a second distension at a constant

inflation rate of 40 mL/min was applied. Subjects were asked to report rectal sensation on the 6-point scale mentioned above.

Symptom assessment

During a 2-week run-in period and during treatment, abdominal pain was assessed by the validated Subject's Global Assessment (SGA) of Abdominal Pain and Discomfort which consists of a 100-mm visual analogue scale with severity descriptors (absent, very mild, mild, moderate, severe and very severe).²⁴

During the treatment period, weekly symptom relief was monitored by the SGA of relief with the following question: compared with the way you felt before entering the study, how would you rate your relief of symptoms during the past week? Possible answers were completely relieved, considerably relieved, somewhat relieved, unchanged or worse.²⁴

The severity of IBS symptoms of diarrhoea, abdominal bloating, flatulence, urgency and the feeling of incomplete evacuation was scored before and after 8 weeks of treatment with the validated Gastrointestinal Symptom Rating Scale, in which the intensities of the symptoms are scored on a 7-graded Likert scale, with descriptive anchors (0 = no symptoms at all; 1 = minimal symptoms; 2 = mild symptoms; 3 = moderate symptoms; 4 = rather serious symptoms; 5 = serious symptoms and 6 = very severe symptoms).²⁵

Data analysis

The primary endpoint was the effect of octreotide on the rectal sensitivity during rectal distension. Secondary endpoints were rectal compliance, global symptom relief, abdominal pain scores and gastrointestinal symptoms.

Rectal compliance was calculated as the slope of the steepest part of the pressure-volume curve during phasic distension, obtained by measuring the mean

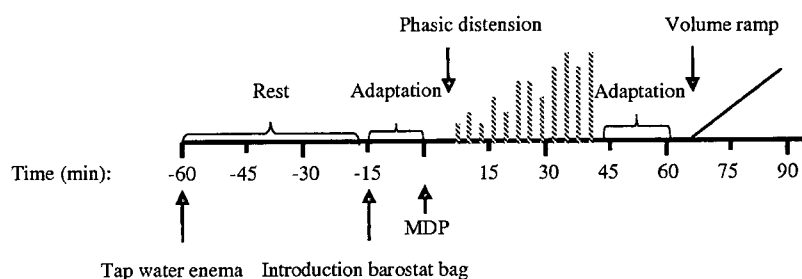


Figure 1. Barostat protocol.

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intrabag volumes over the last 60 s of the first four distension steps and plotting it against the corresponding distending pressure.^{23, 26}

Patients were classified as responder when they reported at least 50% of their weekly SGA of relief assessments as either considerably relieved or completely relieved or 100% at least somewhat relieved.²⁴ The definition of responder in the SGA of abdominal pain and discomfort was a reduction in mean visual analogue scale score of 40%.²⁴ Furthermore, symptom relief is expressed by a weekly percentage of patients reporting some degree of (somewhat to complete) relief of symptoms.

As the expected effect of octreotide on visceral perception would be more pronounced in patients with lower sensory thresholds, we performed a *post hoc* analysis to compare patients with a lower sensory thresholds, or visceral hypersensitivity, to patients with normal rectal sensitivity.

Statistics

The primary endpoint was the threshold for discomfort/pain during rectal distension. Based on the data derived from a previous study,³ we assumed a relevant detectable difference would be 10 mmHg with a common s.d. of 11 mmHg, which is the increase in thresholds needed for IBS-patients to achieve the same level as healthy volunteers. A two group *t*-test with a 0.050 two-sided significance level will have 80% power to detect a difference in means of 10 mmHg, with a common s.d. of 11 mmHg, when the sample sizes in each group is 21. To compensate for potential dropouts, we invited a total of 46 patients to participate in this study protocol.

Statistical evaluations were performed by using commercially available software (SPSS 12.0.1; SPSS

Inc. Chicago, IL, USA). Continuous data were compared by using the Student *t*-test for independent samples and the paired *t*-test for related samples. Nominal data were compared by the Mann-Whitney *U*-test for independent samples or the Wilcoxon signed rank test for related samples. Comparison of proportion was performed by using chi-squared testing. Differences were considered significant at the 5% level. Data are shown in mean $\pm$ SEM.

RESULTS

Study subjects

Table 1 summarizes the demographic characteristics of IBS-patients and the historical control group (healthy volunteers: HV). All participants were Caucasian. Based on their clinical history, 76% of the IBS-patients were considered as having diarrhoea predominant and 24% alternating type of IBS. The mean duration of symptoms at intake was 9.7 ± 1.8 years (range: 0.8–58.0 years). During the treatment period, two participants, both receiving placebo, experienced an increase in abdominal pain and therefore dropped out before finishing the study protocol. Apart from a brief, self-limiting, tenderness at the injection site (in both placebo and octreotide), treatment was well tolerated and no side effects other than IBS symptoms were mentioned.

Rectal sensitivity at baseline

Pressure sensitivity

In IBS patients, the baseline mean MDP level (7.4 ± 0.4 mmHg) did not differ from historical data obtained in healthy volunteers (HV, MDP: 6.3 ± 0.7 mmHg)³.

Table 1. Baseline characteristics				
	HV†	IBS All	Placebo	Octreotide
<i>n</i>	17	46	23	23
Male:female	9:8 (47% F)	22:24 (52% F)	13:10 (43% F)	9:14 (61% F)
Age (years) range	39 $\pm$ 4 (19–62)	38 $\pm$ 2 (19–63)	40 $\pm$ 3 (20–59)	37 $\pm$ 3 (19–63)
% IBS-D	–	76	70	78
Duration of symptoms (years)	–	9.7 $\pm$ 1.8	9.4 $\pm$ 1.8	9.9 $\pm$ 3.1

IBS, irritable bowel syndrome; HV, healthy volunteers (historical control group); IBS-D, diarrhoea dominant IBS.

Data are shown in mean $\pm$ SEM.

†Adapted from Kuiken *et al*³.

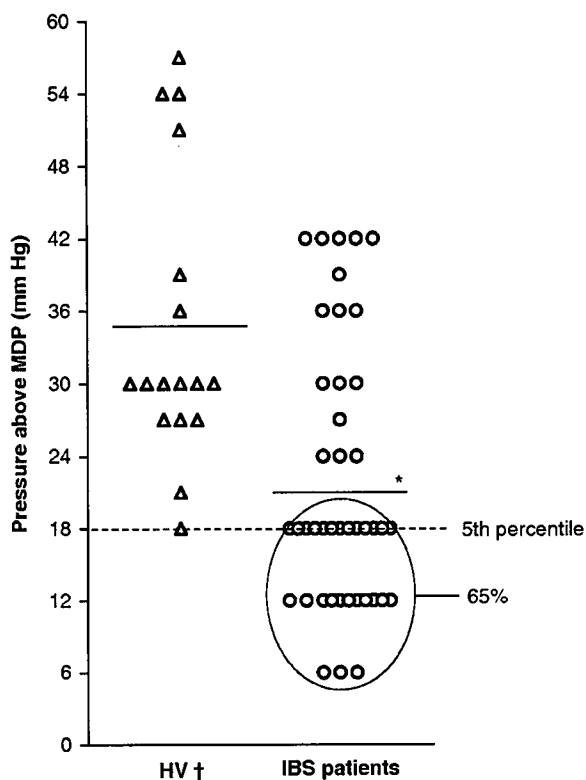
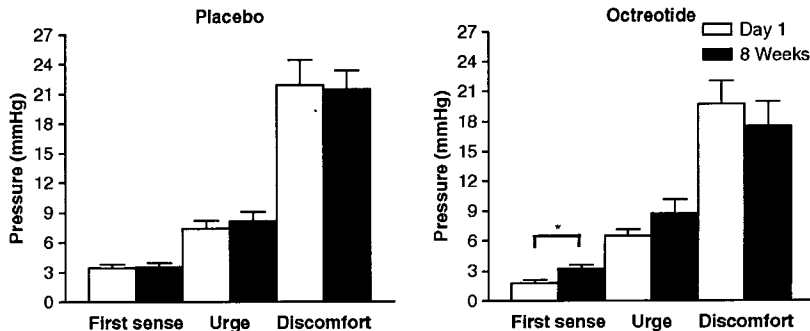


Figure 2. Baseline thresholds for discomfort in mmHg above minimal distending pressure. Mean threshold in healthy volunteers (HV): 35 ± 3 mmHg, irritable bowel syndrome (IBS) 21 ± 2 mmHg. * $P < 0.001$ vs. HV. The upper limit of hypersensitivity is, based on the 5th percentile of HV, defined at 18 mmHg. Of all IBS patients, 30 (65%) were therefore considered to have a hypersensitive rectum. †Adapted from Kuiken *et al.*³

The mean thresholds for first sensation (2.5 ± 0.3 mmHg), urge (6.9 ± 0.5 mmHg) and discomfort (21.0 ± 1.6 mmHg) were all significantly lower than those of healthy volunteers (5.1 ± 1.0 , 12.9 ± 1.3 and 34.8 ± 3.0 mmHg, respectively) ($P < 0.01$)

Figure 3. Effect of placebo and octreotide on rectal sensory thresholds for first sensation, urge and discomfort, before and after 8 weeks of treatment in mmHg above minimal distending pressure. Data are expressed as mean $\pm$ SEM. * $P < 0.001$.



Using the 5th percentile of the discomfort thresholds measured in HV (18 mmHg)³ as cut-off, 30 patients (19 female and 11 male, mean age: 37 ± 2 years) were considered hypersensitive to rectal distension (Figure 2).

Volume sensitivity

During volume-ramp distension, the thresholds in IBS for first sensation, urge and discomfort (55 ± 9 ; 152 ± 14 and 243 ± 14 mL, respectively) were all statistically lower than those in HV (95 ± 23 , 212 ± 24 and 328 ± 20 mL, respectively; $P < 0.01$).

Rectal compliance

The slope of the steepest part of the pressure-volume curve during phasic distension in healthy volunteers was $14 \pm 1 \text{ mL/mmHg}$, which was comparable in IBS patients ($13 \pm 1 \text{ mL/mmHg}$).

Effect of octreotide in IBS patients:

Rectal sensitivity

Pressure sensitivity

Minimal distending pressure levels were not affected by either of the treatment modalities (placebo: before 8.1 ± 0.6 , after 7.8 ± 0.7 mmHg and octreotide: before 7.0 ± 0.7 , after 7.2 ± 0.6 mmHg). Octreotide, but not placebo, significantly increased the pressure sensitivity threshold for first perception. After treatment with octreotide, the mean threshold of first sensation increased from 1.7 ± 0.3 to 3.3 ± 0.4 mmHg above MDP ($P < 0.01$), whereas placebo had no effect (3.4 ± 0.4 vs. 3.6 ± 0.4 mmHg). In contrast, the thresholds for urge or discomfort were not affected by placebo (urge: from 7.4 ± 0.8 to 8.1 ± 1.0 mmHg, discomfort: from 21.9 ± 2.5 to 21.4 ± 1.9 mmHg) or octreotide (urge: from 6.5 ± 0.6 to 8.7 ± 1.4 mmHg, discomfort: from 19.7 ± 2.3 to 17.5 ± 2.5 mmHg) (Figure 3).

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Volume sensitivity

As in pressure-controlled distension, volume thresholds showed a significant increase in mean volume at first sensation during octreotide treatment (44 ± 10 to 77 ± 16 mL, $P < 0.01$), but not during placebo (67 ± 14 to 66 ± 14 mL). No differences were found in threshold for sensation of urge or discomfort in placebo (urge: from 169 ± 20 to 183 ± 20 mL, discomfort: 272 ± 22 , after 286 ± 21 mL) or octreotide (urge: from 136 ± 19 to 146 ± 20 , discomfort: from 216 ± 17 to 220 ± 22 mL).

Rectal compliance

Rectal compliance was not affected by octreotide or placebo. At baseline, compliance was 14 ± 1 and 12 ± 1 mL/mmHg, respectively, in the groups receiving placebo and octreotide. The compliance measured after 8 weeks of treatment, with placebo or octreotide (placebo: 15 ± 1 mL/mmHg and octreotide: 14 ± 1 mL/mmHg), was not different from baseline values.

Hypersensitive vs. normosensitive

Patients with visceral hypersensitivity and patients with normal sensitivity may represent different subpopulations and may respond differently to pharmacologic interventions, especially when targeting visceral sensation. Therefore, we performed a *post hoc* analysis to compare the effect of octreotide on rectal sensitivity in hypersensitive vs. normosensitive IBS patients.

Pressure sensitivity

Both hypersensitive and normosensitive patients showed a small but significant increase in threshold for first sensation after treatment with octreotide (hypersensitive: 1.4 ± 0.4 to 2.7 ± 0.4 mmHg, $P < 0.01$; normosensitive 2.7 ± 0.3 to 5.0 ± 0.6 mmHg, $P < 0.05$) whereas no alterations were observed when treated with placebo (hypersensitive: 3.0 ± 0.6 to 2.8 ± 0.2 mmHg; normosensitive 4.1 ± 0.5 to 4.9 ± 0.8 mmHg) (Figure 4). At baseline, the thresholds for discomfort/pain were comparable in the patient groups. However, octreotide did not significantly alter the mean threshold of discomfort/pain compared with placebo, neither in hypersensitive (13.4 ± 2.4 vs. 16.6 ± 1.5 mmHg) nor in normosensitive (29.3 ± 2.4 vs. 29.0 ± 4.3 mmHg) patients (Figure 5).

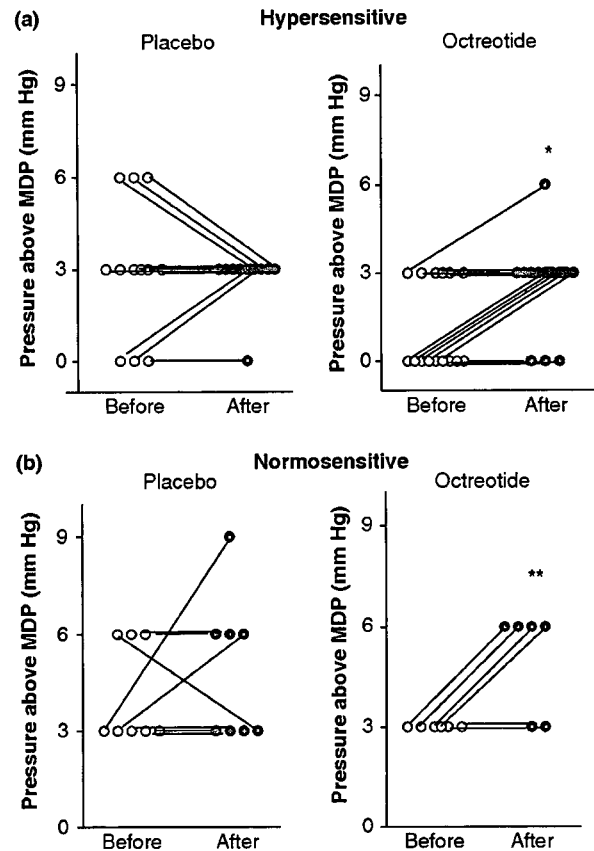


Figure 4. Individual thresholds for first sensation during rectal distension, before and after 8 weeks of treatment with placebo or octreotide. (a) hypersensitive and (b) normosensitive patients. * $P < 0.01$, ** $P < 0.05$, compared to before treatment.

Volume sensitivity

In hypersensitive patients, mean intrabag volume of first sensation was 52 ± 12 mL before, and 45 ± 8 mL after placebo, vs. 28 ± 7 mL and 53 ± 10 mL (NS, $P = 0.05$) with octreotide, respectively. When treated with placebo, the volume of first sensation in normosensitive patients was 90 ± 30 mL before and 101 ± 32 mL after treatment, compared with octreotide, before 89 ± 30 mL and after 147 ± 45 mL (NS). At baseline, the threshold for discomfort/pain measured during volume ramp distension was significantly higher in hypersensitive patients treated with placebo compared with those treated with octreotide (237 ± 24 mL and 182 ± 12 , respectively $P < 0.05$). However, 8 weeks of treatment with octreotide or placebo did not alter these thresholds (placebo 252 ± 24 mL, octreotide 185 ± 21 mL). In the normosensitive patient, baseline

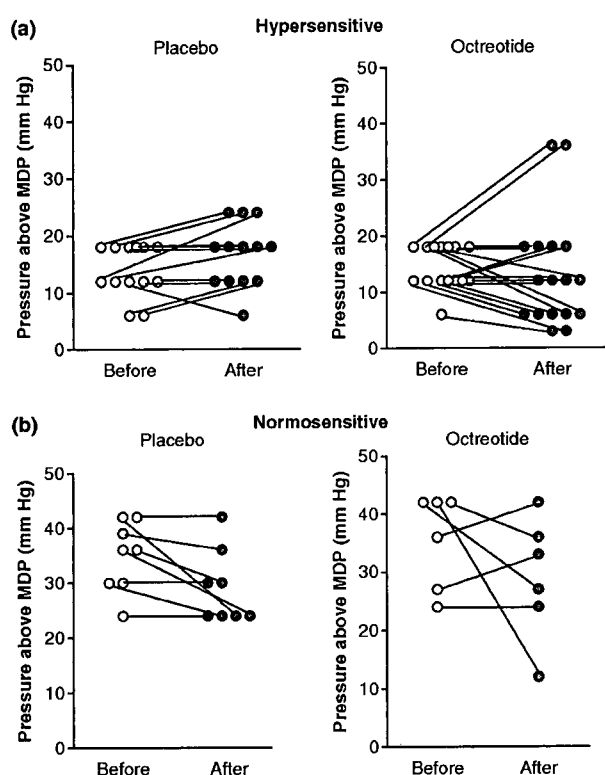


Figure 5. Individual thresholds for discomfort/pain during rectal distension, before and after 8 weeks of treatment with placebo or octreotide. (a) hypersensitive and (b) normosensitive patients.

values were comparable and likewise, no differences were found after treatment with placebo (before: 330 ± 37 mL, after: 341 ± 31 mL, respectively) or octreotide (before: 313 ± 29 mL, after: 318 ± 37 mL).

Rectal compliance

Compliance was comparable between patients receiving placebo or octreotide, before treatment (hypersensitive 13 ± 1 and 12 ± 1 mL/mmHg, respectively; normosensitive 16 ± 2 and 15 ± 1 , respectively) and was not significantly altered by either of the treatment modalities (hypersensitive 15 ± 2 and 14 ± 2 mL/mmHg, respectively; normosensitive 16 ± 2 and 14 ± 1 , respectively).

Symptoms

Global relief

Figure 6 shows the weekly percentage of patients reporting some degree of (somewhat to complete) relief

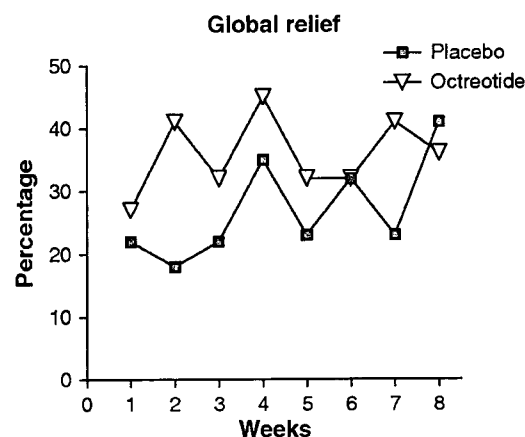


Figure 6. Weekly effect of placebo and octreotide on global symptom relief (SGA of relief), percentage of patients reporting some to complete relief of symptoms.

of symptoms. The percentage of patients experiencing symptom improvement was statistically not different between the two treatment groups. According to the criteria of Muller-Lissner *et al.*²⁴ (at least 50% of the weekly SGA of Relief assessments as either considerably relieved or completely relieved or 100% at least somewhat relieved), one patient is considered responder in the placebo group ($n = 23$) compared with four in octreotide ($n = 23$), which is not significantly different.

Abdominal pain

The mean VAS-scores during screening were comparable between placebo and octreotide treated patients (placebo: 60 ± 4 ; octreotide 57 ± 4 , NS). Treatment with placebo or octreotide did not significantly affect this VAS score of abdominal pain (placebo: 54 ± 4 ; octreotide: 55 ± 5 , NS). After 8 weeks of treatment, one patient was considered a responder (at least 40% reduction in VAS-score) in the placebo-group vs. two patients in the octreotide group (not significant).

Faecal consistency and frequency

Faecal consistency improved significantly during treatment with octreotide. After 8 weeks, the percentage of patients reporting at least moderate complaints of loose stools decreased from 91% to 81% when treated with placebo and from 78% to 52% with octreotide ($P < 0.05$) (Figure 7).

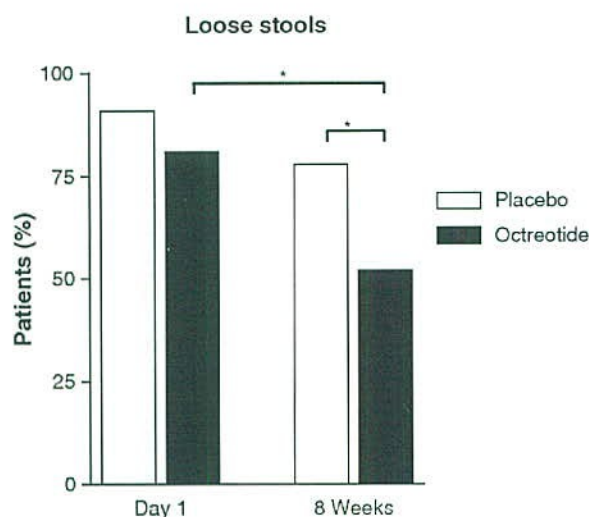


Figure 7. Percentage of patients reporting at least moderate complaints of loose stools at day 1 and after 8 weeks of treatment. * $P < 0.05$.

On the other hand, defecation frequency was not significantly affected by octreotide. The percentage of patients reporting at least moderate complaints of frequent defecation decreased from 91% to 76% with placebo and from 78% to 57% with octreotide (NS) (Figure 8).

Gastrointestinal symptoms

Other IBS symptoms did not improve significantly during treatment with octreotide or placebo (Figure 8).

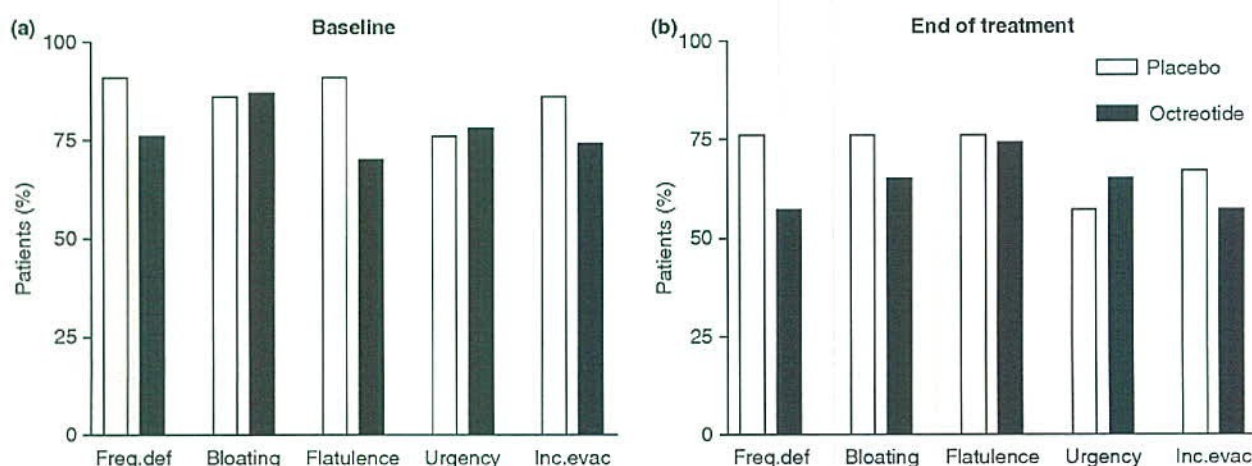


Figure 8. Percentage of patients reporting at least moderate complaints of other irritable bowel syndrome symptoms, obtained by Gastrointestinal Symptom Rating Scale questionnaire. Comparing placebo and octreotide (a) before and (b) after treatment. freq. def, frequent defecation; inc. evac, incomplete evacuation.

Before treatment, the percentage of patients reporting at least moderate complaints of flatulence (placebo 91%; octreotide 70%), bloating (placebo 86%; octreotide 87%), urgency (placebo 76%; octreotide 78%) and the feeling of incomplete evacuation (placebo 86%; octreotide 74%) was comparable between the two treatment groups. At the end of treatment, no significant differences were found in the percentage of patients complaining of flatulence (placebo 76%; octreotide 74%), bloating (placebo 76%; octreotide 65%), urgency (placebo 57%; octreotide 65%) or the feeling of incomplete evacuation (placebo 67%; octreotide 57%).

DISCUSSION

Somatostatin is a peptide hormone with a wide range of activity in the gastrointestinal tract: it inhibits intestinal motility, gastric exocrine and endocrine function, and absorption of nutrients and ions.²⁷ These effects are mediated by somatostatin receptors (SSTR), of which currently five different subtypes have been cloned (SSTR1 to SSTR5).^{28, 29} The expression of SSTR varies enormously in the gastrointestinal tract. For example, all subtypes are expressed in the stomach, whereas in rodents SSTR1 and SSTR5 are found in the small intestines. In human, SSTR2 is expressed in the small intestine and is also found in colon and rectum.³⁰ Mainly based on its anti-secretory effect, octreotide is currently used as treatment of severe diarrhoea. Recent evidence, however, demonstrates

that SSTR may also be involved in visceroperception. In the brain, including in those areas involved in visceral perception, all five receptor subtypes are expressed. In human, epidural administration of somatostatin produces a significant reduction in post-operative pain.³¹ Similarly, in rats, Carlton *et al.*³² demonstrated a reduction of capsaicin-induced pain behaviour following administration of somatostatin, most likely by SSTR2 on neurons in the dorsal root ganglia.

The somatostatin analogue octreotide, which has a high affinity for SSTR2 and SSTR5, has also been shown to have viscerosensory properties. In healthy subjects, octreotide reduces the perception of non-noxious gastrointestinal distensions. However, discomfort or pain was not significantly altered by octreotide.^{10, 12, 33} In contrast, in IBS patients, octreotide has an analgesic effect, as illustrated by an increased rectal threshold of discomfort and pain directly after i.v. or s.c. administration.^{14–16} Based on its visceral analgesic and anti-secretory effect, one might argue that somatostatin analogues could represent a potential new therapeutic approach for non-constipated IBS patients.

So far, studies have only reported on the acute effect of octreotide on visceroperception. However, before accepting octreotide as potential treatment, it is important to investigate whether prolonged administration with octreotide or related agents maintain their effect. This is particularly relevant as chronic administration of SSTR could result in receptor desensitization³⁴. Therefore, in the current study, we evaluated the effect of prolonged treatment with octreotide in patients with non-constipated IBS. Sandostatin LAR is a long-acting slow release preparation of octreotide, used as treatment of acromegaly, gastroentero-pancreatic neuroendocrine tumours and carcinoid tumours. We showed that 8 weeks of treatment with octreotide decreased the sensation of non-noxious rectal stimuli or first sensation. However, no alteration was found in the thresholds of urge and discomfort or pain. Moreover, no effect on maximum tolerated volume or rectal wall compliance was observed, excluding an effect of octreotide on rectal tone. Previously, we demonstrated that patients with visceral hypersensitivity may respond differently to treatment compared with subjects with normal visceroperception.^{23, 35} Based on these findings, one might argue that especially patients with visceral hypersensitivity would react to octreotide. However, a *post hoc* analysis did not reveal a sig-

nificant treatment effect in hypersensitive patients. Therefore, our data suggest that with the current dose, no significant effect on visceral perception (urge/discomfort) can be demonstrated. Similarly, in the current study population and with the current dose of octreotide, we were unable to show clinical improvement: global relief and symptoms of abdominal pain, bloating, flatulence, urgency or the feeling of incomplete evacuation did not improve after 8 weeks of treatment with octreotide. In contrast, stool consistency was significantly improved, a finding in line with its application as treatment of secretory diarrhoea of different aetiologies.²⁰ Possible mechanisms mediating this effect include prolonged gastrointestinal transit time, reduced jejunal secretion and increased water and electrolyte absorption. In addition, as diarrhoea predominant IBS is associated with an exaggerated release of serotonin, reduction of serotonin release by octreotide may contribute as well.³⁶

A possible explanation for the predominant negative findings in this study could be insufficient dosing. The choice of the current dose was based on the treatment schedule of patients with acromegaly, proven to suppress pituitary growth hormone secretion effectively.³⁷ However, higher concentrations may be required to affect visceral sensation. Indeed, peak concentration after octreotide in the acute experiments is considerably higher than the steady-state concentration obtained with Sandostatin LAR. Half-an-hour after 100 µg of octreotide s.c., peak plasma concentration ranges between 2.8 and 5.2 ng/mL. In contrast, after 20 mg of intramuscularly injected Sandostatin LAR, the steady-state concentration of octreotide varies between 1.2 and 2.5 ng/mL.^{38–40} Therefore, higher dosing may have revealed more pronounced effects than 20-mg octreotide used in the current study. Nevertheless, the fact that we did show an effect on first sensation and stool consistency suggests that the current dose was to some extent effective, albeit not optimal.

Secondly, differences in penetration of Sandostatin LAR, because of the relatively low dosage into the central nervous system, could be involved. This may be especially relevant assuming that the analgesic effect of octreotide is mediated by central mechanisms.^{41–43} In human, intrathecal octreotide results in a significant relief of pain caused by cancer.^{42, 43} In addition, Su *et al.* indeed showed a reduction in visceral nociception in rats after intrathecal administration of octreotide.⁴⁴

Thirdly, chronic treatment with an octreotide analogue may lead to tachyphylaxis because of agonist-dependent receptor internalization and desensitization, a well-established phenomenon for SSTR.³⁴ SSTRs undergo internalization in a subtype-selective manner, with SSTR3 and SSTR5 far more prone to be rapidly internalized than SSTR2.³⁷ SSTR2 is proven to be the most important receptor in the treatment of acromegaly, explaining the continued response to treatment in this disorder.⁴⁵ However, we did not observe a reduction in the effect on stool consistency in time, making this explanation less likely.

Finally, it should be emphasized that this study is powered to detect a difference in the threshold of discomfort/pain during rectal distension. Therefore, the number of patients studied is too small to demonstrate

an effect on symptom relief, implying that larger clinical trials with higher doses are required to further evaluate the effect of octreotide on global relief and gastrointestinal symptoms.

In conclusion, this study established the effect of prolonged treatment of non-constipated IBS patients with octreotide. Although the threshold of first rectal sensation increased and stool consistency improved, long-term treatment with octreotide, at least at the current dose used, has no visceral analgesic effect and fails to improve IBS symptoms.

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Review

Epidemiology and Management of Diarrheal Disease in HIV-Infected Patients

Elly T. Katabira, MD

ABSTRACT

Diarrhea is the most common gastrointestinal symptom in human immunodeficiency virus (HIV) infection. It affects up to 90% of patients, becoming more frequent and severe as the immune system deteriorates. It often is associated with significant morbidity and mortality particularly in the developing countries. Gastrointestinal infections, some of which are attributable to inadequate sanitation and poor hygiene are the predominant cause of diarrhea, although multiple medications, including traditional herbs, also may be causes. The basic principles of management include detection of treatable causes, relief of symptoms, prevention of malnutrition, and psychosocial support. In up to 60% of cases, no cause can be identified, partly because of inadequate investigative facilities. Symptomatic treatment is the mainstay of management particularly when no cause can be identified. Unfortunately this can be extremely difficult when the patient is severely immune-suppressed. There is poor response to motility control drugs, such as loperamide, and others, such as octreotide, are too expensive. Fluid replacement should be started early to prevent excessive dehydration. This should be combined with nutritional support to prevent malnutrition. Psychosocial support, including counselling, for both the patient and the caring relatives, is required to alleviate anxiety, particularly when the diarrhea becomes intractable.

Key Words: counselling, diarrhea, education, HIV, nutrition

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Acquired immune deficiency syndrome (AIDS) was first identified in Africa as slim disease, partly because of the associated chronic diarrhea.^{1,2} This is still true in the developing countries particularly in sub-Saharan Africa, where chronic diarrhea lasting for more than 1 month is still an important mode of clinical presentation. It is associated with significant morbidity and mortality, particularly where both financial and human resources are limited to provide adequate care. In this case, effective management calls for a strategic approach involving both conventional and unconventional principles. This article

discusses practical measures suitable for a poor resource setting for managing diarrhea in human immunodeficiency virus (HIV)-infected patients.

EPIDEMIOLOGY

The Scope of Diarrhea in HIV Disease

The prevalence of diarrhea in HIV-infected patients varies from country to country. In some studies, chronic diarrhea has been reported to occur in 40 to 90% of HIV-infected patients.^{3–6} The prevalence tends to be high in communities where there is overcrowding, with its associated poor sanitation.⁵ It also is related to the prevalence of other conditions that usually are associated with diarrhea, such as parasitic infestation. In the AIDS clinic in Mulago Hospital, Kampala, Uganda, 47% of the patients present with diarrhea as one of their symptoms on their first visit.

CLINICAL PRESENTATION

Diarrhea in Early HIV Infection

Whereas chronic diarrhea in HIV infection may be synonymous with a diagnosis of AIDS, many patients may develop bouts of diarrhea from time to time punctuated with long episodes of good health in between. In such cases, it may be difficult to associate this kind of diarrhea with HIV infection, particularly when the patient's HIV serology may not be known. The diarrhea may be triggered by a common nonserious infection or by a reaction to one of the many medications such patients are likely to take.

As the immune status of such a patient is likely to be reasonably good, the recovery is often uneventful. Occasionally, in some patients, this single attack may be severe enough to require hospital admission to correct the electrolyte imbalance that may have occurred. It also may trigger other opportunistic infections, such as oral candidiasis, because of sudden additional depression of the immune system. In this case, the recovery may be protracted, and the patient fails to regain all his weight to the pre-diarrhea status.

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Diarrhea in Late HIV Disease

Clinically diarrhea in late HIV disease presents with chronic, persistent liquid diarrhea, anorexia, nausea, vomiting, loss of energy, abdominal pain or cramps, flatulence, and severe weight loss.⁷⁻⁸ The familiar scenario here is recurrent episodes of diarrhea, lasting for over a month.⁷ In severe cases, the patient may pass between 10 to 20 or more watery stools in a day with associated extreme dehydration. This may further be complicated by development of hemorrhoids, anal fissures, and rectal bleeding. In a patient who develops ischiorectal abscesses and anal fistulas, there may be additional purulent anal discharge.

In terminal stages, the patient loses sphincter control such that there is continuous soiling of the under garments. Even patients who are still mobile, are forced to remain indoors close to toilet facility because of the unpredictability of their diarrhea and the associated odor. This scenario of relentless diarrhea is often associated with rapid and severe dehydration and deterioration of the immune system. As a result of this, the patient develops renal shut down and additional opportunistic infections, such as septicemia or tuberculosis.

DIAGNOSIS

In patients who present with chronic diarrhea, the isolation of the underlying causative agent is possible in only 40 to 60% of cases in sub-Saharan Africa.⁹⁻¹² The success of isolation depends on the sophistication of laboratory facilities. Table 1 presents causes of chronic diarrhea in most patients in sub-Saharan Africa.

Where resources and diagnostic facilities are limited, a thorough clinical evaluation is important. This should include a detailed history to document all drugs and herbs that are being taken at the time or have been, in the recent past; a detailed physical examination to detect any underlying clinical problems that could be responsible for the diarrhea; and a digital rectal examination to exclude fecal impaction, which may be associated with overflow incontinence. Fecal impaction may insidiously

occur in bedridden patients with minimal activity. The integrity of the anal sphincters also should be assessed, because they may be affected in patients with autonomic dysfunction.

MEDICAL MANAGEMENT

General Principles

For any patient who presents with diarrhea at any stage of HIV infection, the following basic principles of care should be adopted^{13,14}:

- 1. Specific treatment of the underlying cause if known
- 2. Fluid replacement
- 3. Management of complications
- 4. Symptomatic treatment of diarrhea
- 5. Nutritional support
- 6. Education of the patient and the caring family about early and appropriate intervention with the view of reducing morbidity and mortality and protecting those caring for the patient
- 7. Psychosocial support for the patient and the caring family.

Specific Treatment of Underlying Causes of Diarrhea

Where a cause can be identified, it should be treated early and aggressively if an appropriate drug is available. Where such a drug is available, such as for *Salmonella* infection, aggressive and prolonged treatment is required. This is because of the patient's impaired immune surveillance, particularly in late HIV disease.

When a patient is taking many drugs and any of them may be responsible for the diarrhea, it is good practice to stop all medications for 24 to 72 hours. This may be followed by a remarkable improvement in the diarrhea. Where it may not be feasible to stop all drugs, a selection of one or two drugs at a time that are likely to be the culprit should be done. Many patients in sub-Saharan Africa take traditional herbs in all sorts of combinations for various reasons. This information may not be volunteered by the patient who may assume that it is not relevant. Stopping such herbs also may help in resolving the diarrhea.

Critical appraisal of the dietary habits of the patient may identify a likely cause of the diarrhea, such as lactose intolerance. Appropriate dietary advice should be given whereby the item likely to cause diarrhea is eliminated from the diet.

Fluid Replacement

Fluid replacement is the cornerstone in the management of fluid loss as a result of diarrhea and vomiting due to any cause. In mild-to-moderate dehydration, oral rehydration may be enough, particularly if it is started early.

Table 1. Causes of Chronic Diarrhea in Patients with HIV Disease	
Infectious Causes	Noninfectious Causes
<i>Cryptosporidium</i>	Medications
<i>Isospora belli</i>	Multiple drug therapy
<i>Microsporidia</i>	Traditional herbs
Giardiasis	Malabsorption
Shigellosis	High osmotic food and drink
Amebiasis	HIV enteropathy
Salmonellosis	Lactose intolerance
<i>Strongyloides</i>	Obstruction with overflow incontinence
Candidiasis	Rectal incontinence
<i>Campylobacter</i>	Stress
Cytomegalovirus	
<i>Mycobacterium avium</i> complex	

Where vomiting coexists with diarrhea, patients should be instructed to take small amounts of oral fluids frequently (a tablespoonful or two at a time). Large amounts taken in one dose may induce further vomiting because of sudden distention of the stomach. The ideal fluid should be freshly prepared from oral dehydration salts sachets. Where these are not available, any fluid will do, as long as it is hygienically prepared.

In most African countries, passion fruit, oranges, and other fruits are readily and cheaply available. Their preparations should contain a small amount of added sugar to emulate the commercially prepared oral dehydration salts. Too much added sugar is likely to make the diarrhea worse. Where such fruits are not available locally, prepared solutions of 8 teaspoons of sugar and 1 of table salt in one liter of boiled but cool water should be given.

In severe dehydration, parental fluids should be started early and given aggressively but cautiously. In severely emaciated patients, fluid overload can easily occur, particularly when central line venous pressure monitoring facilities are not available. While the patient is receiving parental fluids, oral fluids also should be introduced early and continued thereafter. This will encourage early mobilization and reduce the risks of prolonged immobilization.

Management of Complications

Patients with severe diarrhea in late HIV disease may develop profound dehydration, which may lead to a number of complications, including electrolyte abnormalities, particularly hypokalemia. This results in severe weakness and hypotonia. Where facilities are available, serum electrolyte levels should be obtained. The hypokalemia should be corrected by potassium chloride (KCl) supplements. Where KCl is not available, oral rehydration salts (ORS) and local juices may help. Most local juices, such as those from passion fruit and oranges contain a reasonable amount of potassium. Fortunately, early intervention in correcting the dehydration by appropriate intravenous fluids can prevent such complications from developing.

The morbidity associated with severe dehydration also may be complicated by the development of other infections, such as severe oral and esophageal candidiasis, septicemia, and multiple abscesses. Antifungal agents, such as ketoconazole, should be given when oral and esophageal candidiasis is present. When septicemia is suspected, blood cultures should be done, if facilities are available, otherwise broad-spectrum antibiotics should be started. Where abscesses are identified, these should be incised, drained, and cultured if facilities are available.

Symptomatic Treatment of Diarrhea

In over 60% of cases, no specific cause of diarrhea can be identified.⁹ In this case, management consists of

symptomatic control of diarrhea. The drugs often used include adsorbent and bulk-forming types, such as kaolin, and antimotility drugs such as loperamide. Kaolin is readily available and inexpensive, but unfortunately, it is not usually very effective in chronic diarrhea. Patients may be advised to increase their fiber content in their diet, although this often makes food less palatable, particularly in the presence of anorexia. Antimotility drugs occasionally may relieve the associated abdominal cramps and even stop the diarrhea, particularly in those with early HIV disease. However, in chronic diarrhea their usefulness is often limited. In many cases, the benefit obtained from such drugs is short-lived, and patients end up trying them all without any long-lasting success. Some of these drugs do cause variable sedation, which may make the patient appear weaker to the caring family. Newer agents, such as octreotide, are too expensive and are not readily available.

Nutritional Support

Persistent diarrhea often is associated with malnutrition. This may arise in several ways. Food intake is often limited because of the associated nausea and vomiting. Some of the nutritious foods may themselves cause diarrhea and therefore have to be abandoned. The underlying cause of the diarrhea itself may disrupt the intestinal mucosal surface so that the absorption of nutrients is impaired.

Nutritional support implies that there should be close interaction with a nutritionist, to identify what foods are suitable, available, and affordable by the patient. Where resources and facilities are available, parental nutrition should be considered. In addition to available foods, vitamin supplements are commonly used together with artificially prepared foods. Unfortunately the latter tend to be expensive and out of reach to many patients.

Patient Education

In resource-poor settings where both drugs and qualified health professionals are in short supply, educating patients in the management of their own diarrhea is a good investment. Patient education should cover the following:

- The significance of diarrhea in their HIV disease
- The common causes of diarrhea and how they can be prevented through improved hygienic practices, such as regular washing of hands before eating anything
- Basic principles of effective control and management of diarrhea
- Guidelines on preparing oral rehydration solutions using ORS sachets or any other locally available material

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- How to recognize and assess the severity of diarrhea and when to seek medical attention from available health facilities
- General care as it relates to diarrhea, including perianal care, use of incontinent devices to prevent soiling and reduction of the foul odor related to frequent diarrhea by various deodorization techniques.

Similar education also should be given to the caring families. In addition, the family should be educated on how to care for the moribund patient and make him comfortable in spite of the continuous diarrhea. Simple things like use of a plastic sheet to protect the beddings; use of paddings or any other available incontinent devices (e.g., specially designed pants) may encourage the patient to be mobile. However, these need to be changed regularly to reduce the risks of soreness.

The people caring for patients with chronic diarrhea should also be educated on how to protect themselves from accidental HIV infection through contact with the patient's body fluids, particularly fecal contamination. They should always wash their hands with soap and water after each time they come in contact with the patient. It also is good practice for them to wash their hands before they attend to the patient so as not to introduce infections to the susceptible patient. They should use gloves when cleaning the patient or changing soiled beddings. Where gloves are not available, the attendant should use another clean, dry piece of cloth as a hand protection while cleaning and removing soiled linen. Attendants with abrasions and open wounds on their hands should ask someone else to do the cleaning if they do not have gloves.

Psychosocial Support

Human immunodeficiency virus disease is a chronic problem in which psychosocial support plays a major part in the complete management of the patient. Support can be achieved through counselling both the patient and the caring family. Counselling opportunities should be provided on a regular basis and at any time of contact with a health professional. During counselling sessions, various patient concerns, such as coping emotionally with the diarrhea and other relevant issues should be discussed. Issues need not be only health-related; they may include other things, such as job and family welfare, particularly with regard to children. These issues may be a constant source of worry to the patient, which may interfere with the subsequent management of the diarrhea.

Many caring families in sub-Saharan Africa go through unrecognized and unappreciated distress as they care for

their loved ones with relentless diarrhea. Well-timed counselling to them goes a long way in alleviating stress so that they are able to cope with the difficult task of caring for these patients. In the end it is the patients who benefit most.

CONCLUSION

Chronic diarrhea is a major cause of morbidity in an HIV-infected patient. The approach to its management should be comprehensive. It should address not only the causes of diarrhea and its complications but also the patient as a whole, including the caring family and other care givers.

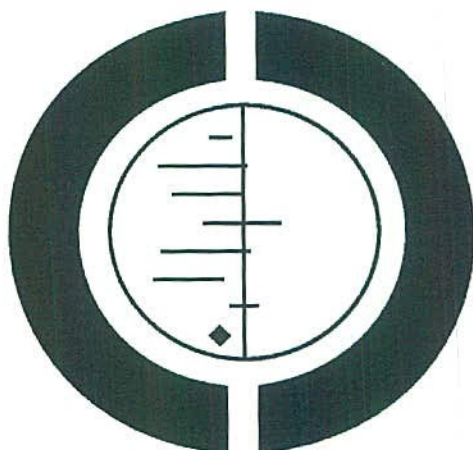
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Antimotility agents for chronic diarrhoea in people with HIV/AIDS (Review)

Nwachukwu CE, Okebe JU



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Antimotility agents for chronic diarrhoea in people with HIV/AIDS (Review)
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[Intervention Review]

Antimotility agents for chronic diarrhoea in people with HIV/AIDS

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ABSTRACT

Background

AIDS-related diarrhoea is a common cause of morbidity and mortality in HIV positive individuals, especially in the sub-Saharan Africa where 70% of deaths from HIV occur. It often compromises quality of life both in those receiving antiretroviral therapy (ART) and the ART naive. Empirical antidiarrhoeal treatment may be required in about 50% of cases which are non-pathogenic or idiopathic and in cases resulting from antiretroviral therapy. Antimotility agents (Loperamide, Diphenoxylate, Codeine) and adsorbents (Bismuth Subsalicylate, Kaolin/Pectin, Attapulgit) are readily available, and have been found to be useful in this condition and so, are often used. Antimotilities are opioids, decreasing stool output by reducing bowel activity thereby increasing fecal transit time in the gut, promoting fluid and electrolyte retention while adsorbents act by binding to fluids, toxins and other substances to improve stool consistency and eliminate the toxins. Due to its potential impact on the management of chronic diarrhoea in persons with HIV/AIDS, we reviewed the effectiveness of antimotility agents in controlling chronic diarrhoea in immunocompromised states caused by HIV/AIDS.

Objectives

To assess the effectiveness of antimotility agents in controlling chronic diarrhoea in people with HIV/AIDS.

Search methods

We searched Medline, EMBASE, the Cochrane Controlled Trials Register, the Cochrane HIV/AIDS Register and AIDSearch databases in November 2006. We also contacted WHO, CDC, pharmaceutical companies and experts in the field for information on previous or on-going trials and checked reference list from retrieved studies, irrespective of language and publication status.

Selection criteria

Randomised controlled trials comparing an antimotility agent or an adsorbent with another antimotility agent, placebo, an adsorbent or no treatment in children and adults diagnosed with HIV and presenting with diarrhoea of three or more weeks duration.

Data collection and analysis

Two authors independently undertook study selection and examined full articles of potentially eligible studies.

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Main results

One trial was found assessing the use of an adsorbent (attapulgate) compared to a placebo for chronic diarrhoea in people with HIV/AIDS. It included 91 adults (Aged 18 to 60), diagnosed with AIDS and experiencing diarrhoea for at least 7 days. There was no evidence that attapulgate is superior to placebo in controlling diarrhoea by reducing stool frequency and normalising stool consistency on days 1 (0.34 (95% CI 0.01 - 8.15)), 3 (1.35 (95% CI 0.51 - 3.62)) and 5 (1.74 (95% CI 0.89 - 3.38)). This was a small trial and may not have had enough power to show evidence of effects. Five deaths were reported which was not classified according to the arms of the study.

Studies assessing the use of antimotility agents were not found.

Authors' conclusions

This review highlights the absence of evidence for the use of antimotility agents and adsorbents in controlling diarrhoea in people with HIV/AIDS. While no trials assessing the use of Antimotilities were found, the retrieved study showed that attapulgate was not better than placebo in controlling diarrhoea in HIV/AIDS patients. For optimum patient care, these agents can still be used, with greater emphasis placed on adjunct therapies like massive fluid replacement while evidence for practice is awaited from further studies and reviews.

PLAIN LANGUAGE SUMMARY

Use of antimotility drugs (Loperamide, Diphenoxylate, Codeine) to control prolonged diarrhoea in people with HIV/AIDS.

People with HIV/AIDS often develop prolonged diarrhoea which are sometimes not caused by infections. This is more so in the sub-Saharan Africa where drugs for controlling HIV itself i.e. antiretroviral drugs (ARV) may not be widely available or affordable. prolonged diarrhoea often results in prolonged illness and death due to loss of fluids, if not treated effectively and on time. Antimotility drugs and adsorbents are readily available and are used to try to control this condition while efforts are made to receive ARVs. We did not find enough evidence to support or refute their use in controlling this condition.

BACKGROUND

Human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) is a global pandemic and has been reported in almost every country of the world. About 66% (22 million) of the estimated 33 million infected people are in sub-Saharan Africa and an estimated 1.3 million children under 15 years are living with HIV/AIDS (WHO 2008). Of the nearly 3 million HIV/AIDS deaths that occurred globally in 2005, 2 million (70%) were in sub-Saharan Africa (UNAIDS 2006).

HIV/AIDS is predominantly a sexually transmitted infection but it is also transmitted by blood and blood products and vertically from infected mothers to their children. It presents with a lowered ability to resist infections (immunodeficiency) resulting from a progressive depletion in the appropriate component of white blood cells (CD4 T-lymphocytes) from direct infection and destruction of these cells by HIV. This predisposes the individual to a myriad of opportunistic infections. The common opportunistic infections associated with HIV/AIDS are *pneumocystis jiroveci*

(formerly carinii) pneumonia, tuberculosis (TB), bacterial pneumonia, cryptococcal meningitis, unexplained chronic diarrhoea, toxoplasmosis, oral candidiasis, etc (Kaplan 2004; WHO 2005). From the current classification of HIV/AIDS, people with CD4 cell counts less than 350/ μ L are at high risk of developing opportunistic infections (WHO 2005) with the more severe ones occurring when counts are below 200/ μ L. Chronic diarrhoea and HIV wasting syndrome appear more common in low income settings (Deschamps 2000).

About 90% of persons living with HIV/AIDS develop diarrhoea of more than seven days at sometime. This can lead to, dehydration, significant weight loss, malnutrition, malabsorption and can result in increased mortality. It may exacerbate the immunodeficiency state (Pape 1994; Edwards 1990).

Fifty to 60 percent of cases of diarrhoeal in HIV/AIDS patients are due to an identifiable infection (Carcamo 2005). Common

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isolates include bacteria and parasites although some may have infections with multiple organisms (Katabira 1999; Pape 1994; Gassama 2001). Some studies show non-pathogenic or unexplained diarrhoea in about 50% of cases (Dryden 1988; Mwachari 1998). This results from malignancies (Kaposi sarcoma, lymphoma), (O'Brien 2003; Sherman 2000), malabsorption, idiopathic causes (possibly from HIV infection) (WHO 1991) and side effects of antiretroviral therapy (ART) especially those containing protease inhibitors which may reduce adherence (Moyle 1998; Sherman 2000; Carr 2000). This is suspected to be as a result of inflammation in the gut, drug molecules attracting fluid into the gut lumen and an overall increased fluid secretion from the gut walls secretory effect (Jamjian 2000)

In high income countries, the use of ART has reduced the incidence of these opportunistic infections (Anastasi 2000; UNAIDS 2004). Diarrhoea remains an important cause of morbidity and mortality in people with HIV/AIDS in low income countries where these drugs are either not readily available or affordable (Logie 2004). Many of the affected people are poor and live in rural areas with ill-equipped primary care centres with limited access to simple drugs (Krain 2005; UNAIDS 2004), so that there is poor access to adequate healthcare and laboratory facilities.

Management of diarrhoea in HIV/AIDS requires a step-wise multi-disciplinary approach for aggressive treatment while at the same time decreasing patient discomfort and cost. This ideally involves the use of ART as well as interventions for the specific underlying conditions to improve the length and quality of life. Severe diarrhoea is largely treated by massive fluid replacement, specific antimicrobials for infective diarrhoea confirmed by stool culture and drug sensitivity studies, antidiarrhoeal agents and nutritional support (DuPont 1993; Guarino 2004). The approach to HIV/AIDS related diarrhoea in high income countries aims at investigations and treatments to isolate and eradicate specific infecting organisms (Walter-Smith 1993; Guarino 2004). In sub-Saharan Africa and other resource limited areas, this is often not possible owing to constraints of technology, human resource and funds. Therefore, empirical treatment remains a good option in these areas while technology is advancing. Opinions however vary on the best treatment modalities. In patients with HIV/AIDS and chronic diarrhoea, varying degrees of efficacy has been reported following use of ART (Foudraine 1998; Silverberg 2004), antibiotics (Guest 2004; Kadapu 2002), alternative medicine (Anastasi 2003; Cohen 1999). A recent review highlighted a number of Chinese herbs used to reduce morbidity and mortality in HIV/AIDS patients (Liu 2005). Other therapies used include combinations of antibiotics and anti-diarrhoeal agents (WHO 1991) and anti-diarrhoeal agents (including antimotility agents, adsorbents, calcium carbonate, octreotide) alone (Schiller 1982; Compean 1994;

Turner 2004).

Antimotility drugs and adsorbents have been found to be of use in controlling diarrhoea (Schiller 1982; Dumitrascu 2004; WHO 1991; McQuain 2004) including in people with HIV/AIDS (WHO 1991; Sherman 2000) and diarrhoea caused by ART (Carr 2000) but should not replace the use of anti infective agents. Antimotility drugs are opioid in action, decreasing bowel activity and increasing tone in the large intestine. This increases fecal transit time allowing absorption of fluid from stool into the gut, thus decreasing stool output (Schumacher 2004; Greenwood 2004). This action also help retain electrolytes and improve hydration. The commonest opioids used against diarrhoea are synthetic such as loperamide (Imodium®), diphenoxylate (Lomotil®) and an opium alkaloid, codeine (Generic) (WHO 1991). Morphine is also used, more so in terminal cases. Associated adverse effects include nausea, vomiting, dry mouth, constipation, respiratory depression and effects on the central nervous system (CNS) such as sedation, anxiety, confusion and tolerance. Diphenoxylate crosses the blood brain barrier (BBB) sparingly and so along with codeine are progressively more sedative, addictive and prone to dependence and abuse. Loperamide does not cross the blood brain barrier, therefore the CNS effects are not seen and so it is not subject to abuse. The use of antimotility agents is limited in children (Armon 2001) and in those with bloody diarrhoea as they may precipitate toxic megacolon in ulcerative colitis or prolong duration of diarrhoea with shigella or salmonella infection (Schumacher 2004). Where this is suspected, they are used in conjunction with antibiotics, especially in adults. Adsorbents (Bismuth Subsalicylate, kaolin/Pectin, Attapulgit, etc) are known to be less effective but more active in acute cases of diarrhoea than antimotilities (McQuain 2004). They act by adsorbing fluids and compounds and binding potential intestinal toxins to improve stool consistency and eliminate these toxins from the gut. Side effects of adsorbents are rare and may present as dark stools and constipation which are dose dependent.

Due to the impact of chronic diarrhoea on morbidity and mortality on persons with HIV/AIDS, we review the effectiveness of antimotility agents in controlling chronic diarrhoea in the immunocompromised states caused by HIV/AIDS.

OBJECTIVES

To assess the effectiveness of antimotility agents in controlling chronic diarrhoea in people with HIV/AIDS.

METHODS

Criteria for considering studies for this review

Types of studies

Randomised controlled trials.

Types of participants

People (children and adults) diagnosed as HIV positive presenting with diarrhoea of three or more weeks duration. Diarrhoea was defined as passage of three or more loose stools in 24 hours (DuPont 1992).

Types of interventions

Intervention

Antimotility drug or an adsorbent

Control

Another antimotility drug, an adsorbent, placebo or no treatment. Fluid replacement therapy should be identical in both groups. Anti infective agents might be given, where indicated.

NB: We excluded trials investigating the use of cannabis (marijuana) because of an ongoing Cochrane Review (Lutge 2005)

Types of outcome measures

Primary

1. Control of diarrhoea (Measured by stool frequency, stool volume and stool consistency on days 1, 3, 7, 14, 28 and 42).

Secondary

1. Dehydration (In children - Significant/not significant - see Table 01 (Adelman 1996))
2. Quality of life (Using a self reporting scoring system such as, but not limited to, Karnofsky score (O'Dell 1995))
3. Adverse events
 - Severe adverse events (Death, leading to hospitalisation)
 - Adverse events leading to discontinuation of therapy
 - Other adverse events

Search methods for identification of studies

See: HIV/AIDS Group methods used in reviews.

See: HIV/AIDS Cochrane Review Group search strategy.

Electronic Searches

Assisted by the HIV/AIDS Review Group Trials Search Coordinator, we developed a comprehensive and exhaustive search strategy in an attempt to identify all relevant studies regardless of language or publication status (published, unpublished, in press and in progress). Full details of the Cochrane HIV/AIDS Review Group methods and the journals hand-searched are published in *The Cochrane Library* in the section on Collaborative Review Groups (<http://www.mrw.interscience.wiley.com/cochrane/clabout/articles/HIV/frame.html>).

We used the RCT search strategy developed by The Cochrane Collaboration and detailed in the Cochrane Reviewer's Handbook, in combination with terms specific to antidiarrhoeals. We searched the following electronic databases in January 2006:

(1) Medline (1966-2005) via PubMed using the following strategy:
#1 Search HIV Infections[MeSH] OR HIV[MeSH] OR HIV[tw] OR hiv-1*[tw] OR hiv-2*[tw] OR hiv1[tw] OR hiv2[tw] OR HIV infect*[tw] OR human immunodeficiency virus[tw] OR human immunodeficiency virus[tw] OR human immune-deficiency virus[tw] OR ((human immun*) AND (deficiency virus[tw])) OR acquired immunodeficiency syndrome[tw] OR acquired immunodeficiency syndrome[tw] OR acquired immuno-deficiency syndrome[tw] OR acquired immune-deficiency syndrome[tw] OR ((acquired immun*) AND (deficiency syndrome[tw])) OR "Sexually Transmitted Diseases, Viral"[MeSH:NoExp]

#2 Search randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized controlled trials [mh] OR random allocation [mh] OR double-blind method [mh] OR single-blind method [mh] OR clinical trial [pt] OR clinical trials [mh] OR ("clinical trial" [tw]) OR ((singl* [tw] OR doubl* [tw] OR trebl* [tw] OR tripl* [tw]) AND (mask* [tw] OR blind* [tw])) OR (placebos [mh] OR placebo* [tw] OR random* [tw] OR research design [mh:noexp] OR comparative study [mh] OR evaluation studies [mh] OR follow-up studies [mh] OR prospective studies [mh] OR control* [tw] OR prospectiv* [tw] OR volunteer* [tw]) NOT (animals [mh] NOT human [mh])

#3 Search DIARRHOEA OR DIARRHEA

#4 Search ANTI-MOTILITY AGENTS OR ANTIMOTILITY AGENTS OR ANTI MOTILITY AGENTS OR GASTROINTESTINAL AGENTS OR GASTRO-INTESTINAL AGENTS OR GASTRO INTESTINAL AGENTS OR ANTIDIARRHEALS OR ANTI-DIARRHEALS OR ANTI DIARRHEALS OR ANTIDIARRHOEALS OR ANTI-DIARRHOEALS OR ANTI DIARRHOEALS OR LOPERAMIDE OR DIPHENOXYLATE OR CODEINE OR IMODIUM OR LOMOTIL OR ADSORBENTS OR BISMUTH SUBSALICYLATE OR KAOLIN OR PECTIN OR KAOLINPECTATE

#5 Search #1 AND #2 AND #3 AND #4

#6 Search #1 AND #2 AND #3 AND #4

This yielded 60 records of which none was selected for full article retrieval.

(2) EMBASE (1980-2005) using the Pubmed strategy modified for EMBASE. This yielded 27 records of which none was selected for full article retrieval.

(3) The Cochrane Library Controlled Trials Register (CLCTR). This contains mainly reference information to randomised controlled trials and controlled clinical trials in health care. This yielded 67 records in total of which none was selected for full article retrieval.

(4) AIDSearch (1980-2005) which includes coverage of the fol-

lowing conferences:

- International AIDS Conference
- Conference on Retroviruses and Opportunistic Infections
- The British HIV Association conference
- International Congress on Drug Therapy in HIV Infection

Searching other resources

This yielded 39 records of which one was selected for full article retrieval but did not meet the inclusion criteria (See table of excluded studies).

There was handsearching of hard copy databases. This yielded one record which was eligible for inclusion in the review (Ilboudo 1997).

We also contacted the World Health Organisation (WHO), Centres for Disease Control (CDC), pharmaceutical companies and experts in the field for information on previous or on-going trials and checked reference list from retrieved studies, without any language restrictions. This produced one study which did not qualify for eligibility assessment.

Data collection and analysis

Selection of trials

Two authors (CN and JO) independently undertook study selection. Full articles of potentially eligible studies were also compared and examined independently by both authors and there were no disagreements on the eligibility or otherwise of the selected study. CN contacted authors of trials for missing information.

Assessment of methodological quality

Risk of bias was assessed as follows:

(1) Randomisation

Selection bias was assessed by the method of generation of allocation sequence and adequacy of allocation concealment, classified 'Yes' if adequate, 'No' if not adequate and 'Unclear' if not well described by the authors in such a way that its adequacy is describable.

(2) Blinding

Performance and detection biases were assessed by checking whether participants, investigators or assessors were blinded, classified 'Yes' if adequate, 'No' if not adequate and 'Unclear' if not well described by the authors in such a way that its adequacy is describable.

(3) Inclusion of all randomised participants.

Attrition bias was assessed by the adequacy of follow up and intention to treat analysis. Trials with loss to follow of less than 10% were rated as adequate (Yes), inadequate (No) if more than 10% and unclear if not well described by the authors in such a way that its adequacy is describable.

Data collection

For the included trial, we collected information, with a standard data collection form, regarding location of the study, methods of the study (as per quality assessment checklist), characteristics of the participants (age range, eligibility criteria), and types of interventions and outcomes. CN contacted authors for missing data and information. CN and JO performed data extraction independently and the review mentor (KG) resolved any disagreement. Dehydration was planned to be assessed as mild, moderate or severe with the following physical signs and symptoms (Adelman 1996):

Mild - alert, normal skin elasticity, normal pulse, thirsty, restlessness.

Moderate - lethargic, thirsty, slow skin retraction, sunken eyes, rapid and weak pulse, sunken anterior fontanelle, deep and may be rapid respiration.

Severe - Drowsy, may be unconscious, very slow skin retraction, wrinkled skin, cold clammy extremities, rapid feeble pulse, grossly sunken eyes, absent tears, deep and rapid respiration.

Significant dehydration was to be taken as moderate/severe dehydration. Data was not available to assess dehydration.

Analysis

We collected each dichotomous outcome for effect in terms of risk ratio with a 95% confidence interval. Continuous outcomes were assessed with a weighted mean difference (WMD) and 95% confidence interval. We planned to assess for heterogeneity by checking for overlapping of confidence interval in the forest plot, test with Cochrane Q test and I^2 test for possibilities of statistically pooling of the outcomes from different studies as well as conducting a sub-group analysis on the following sub-groups: Age (children and adults), concomitant use of ARVs, protease inhibitor containing and non protease inhibitor containing ART and concomitant use of any other antidiarrhoeal therapy. There were, however, no sufficient studies or data to carry these out.

RESULTS

Description of studies

See: Characteristics of included studies; Characteristics of excluded studies.

The initial search (January 2006) identified 113 potentially relevant titles. The review was later expanded to include adsorbents in the interventions with a new search (November 2006) identifying 147 potentially relevant titles. After reviewing the titles and abstracts, 2 papers in full text were obtained for consideration of inclusion into the review. One paper (Compean 1994) was excluded as detailed in the table "characteristics of excluded studies" for not comparing an antimotility agent or an adsorbent with another antimotility agent, an adsorbent, placebo or no treatment.

The review identified 1 eligible study (Ilboudo 1997) as detailed in the table "characteristics of included studies".

Participants

The included study was based on treatment of chronic diarrhoea in people with HIV/AIDS randomised to receive either Attapulgitte or placebo. Ninety-one adults aged 18 - 60 years from 6 university hospitals in 5 African French speaking countries were included; 45 participants were assigned to attapulgitte and 46 assigned to receive placebo. The duration of the study was not clearly stated in the report. Participants were included if they had AIDS diagnosis by CDC criteria, diarrhoea for at least 7 days (at least 4 loose stools per day) and eligible for at least 7 days of hospitalization.

Those receiving other therapies which might influence the study drugs (antacids, modifiers of the transit, antisecretory and antibacterial drugs) were excluded.

Interventions

Participants were randomised to receive 6g of Attapulgitte in 30cl water or matching placebo in 3 divided doses for 5 consecutive days. Oral rehydration was given where necessary, diarrhoeal drugs (not clearly described) were also prescribed.

Outcomes

This review's primary outcome is resolution of diarrhoea measured as stool frequency, volume and consistency repeatedly. The report of the study was only sufficient to record data for stool frequency and consistency on days 1, 3 and 5; day 5 record was, however, not defined in the protocol but was included so as not to lose valuable data. Stool frequency was defined as the number of participants with frequency less than 3 stools per day while stool consistency was defined as number of participants with formed stool after 5 days of treatment. Of the secondary outcomes, only adverse events were recorded, though without sufficient data to be included for analysis.

The study did not report details of randomization, dates and duration of the trial, source of funding and ethics approvals, details of other treatments given and undesirable effects. Efforts to reach the authors for further information were not successful.

Risk of bias in included studies

Methods of sequence generation, allocation concealment and blinding were not well explained. The risk of bias is therefore unclear.

All randomised participants were included in the analysis as there were no losses to follow-up. There is therefore a low risk of bias.

Effects of interventions

One trial was found assessing the use of an adsorbent for chronic diarrhoea in people with HIV/AIDS. It included 91 adults (Aged 18 to 60 years), diagnosed with AIDS and experiencing diarrhoea for at least 7 days.

Comparison: Attapulgitte versus placebo.

Stool frequency.

The fixed effects summary estimate of the relative risk for stool frequency in the study (Ilboudo 1997) for days 1, 3 and 5 were 0.34 (95% CI 0.01 - 8.15), 1.35 (95% CI 0.51 - 3.62) and 1.74 (95% CI 0.89 - 3.38) respectively. These show no evidence of effectiveness of attapulgitte compared with placebo on stool frequency in any of the days.

Stool consistency.

The fixed effects summary estimate of the relative risk for stool frequency in the study (Ilboudo 1997) for days 1, 3 and 5 were 0.34 (95% CI 0.01 - 8.15), 1.35 (95% CI 0.51 - 3.62) and 1.74 (95% CI 0.89 - 3.38) respectively. These show no evidence of effectiveness of attapulgitte compared with placebo on stool consistency in any of the days.

Adverse events.

The study reported 5 deaths. There was however no explanation on the follow up period or on which arm of the study they occurred. The small number of participants used in this study may not be sizeable enough to show evidence of effects.

DISCUSSION

The lone study included in this review is characterised by some methodological flaws in terms of paucity of information given. There was, however, no demonstrable evidence that the antidiarrhoeal agent studied is superior to placebo in controlling diarrhoea in participants. This may not be surprising because of the small sample of participants used for this study.

Review articles and trials comparing antimotilitytics with other anti diarrhoeal agents report varying levels of effectiveness. A randomised controlled trial comparing Octreotide (a somatostatin analogue) and a combination of Loperamide and Diphenoxylate in HIV positive individuals showed Octreotide as more effective in reducing bowel movement (mean bowel movement per day= 2.1 versus 7; $P<0.05$) (Compean 1994). A study comparing Loperamide to Racecadotrin (an enkephalinase inhibitor) showed no difference in mean duration of diarrhoea (13 hours versus 19.5 hours; $P=0.23$) in individuals who were HIV negative (Wang 2005). Loperamide had been demonstrated to be effective in controlling diarrhoea in populations without consideration of their sero-status (Brawner-Caluag 1993). This effect may not be as much in HIV positive individuals due to immune-depression, higher severity of diarrhoea and the wasting syndrome associated with HIV/AIDS.

The use of antimotility agents and its recommendations in treatment guidelines (WHO 1991) for controlling diarrhoea in HIV positive people are a result of extrapolations from studies carried out on HIV negative persons and from expert opinion. Trials assessing the effectiveness of antimotility agents and adsorbents for

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chronic diarrhoea in people with HIV/AIDS are necessary. This may be conducted on individuals on ART in resource limited areas with the view that the evidence generated can be used on those not receiving ART pending when they will have access to ART.

This search for evidence will need to be extended to conducting trials and systematic reviews comparing antimotility agents with other antidiarrhoeals, weighing benefits against cost, at least, which will be helpful even when ART becomes universally available.

AUTHORS' CONCLUSIONS

Implications for practice

This review highlights the absence of evidence for the use of antimotility agents and adsorbents in controlling diarrhoea in people with HIV/AIDS. The poorly-reported single study included show that Attapulgit (an adsorbent) was not better than placebo in controlling diarrhoea in HIV/AIDS patients while no trials assessing the use antimotilitic were found. For optimum patient care, these agents can still be used, with greater emphasis placed on adjunct therapies like massive fluid replacement (Abubakar 2007). Though their effectiveness have not been validated, other antidiarrhoeals like Octreotides can also be used by clinicians while awaiting the evidence of their effectiveness from further studies and reviews.

Implications for research

Trials assessing the effectiveness of antimotility agents and adsorbents in controlling chronic diarrhoea in HIV positive individuals

in resource limited settings are needed. Since it would be unethical to carry out this study in people with HIV/AIDS without providing treatment for control of the disease, it may fit in a module evaluating adjunct therapies in those on ARVs with chronic diarrhoea. Considerations should also include cost benefit analysis and availability.

To provide for alternative regimens, trials and systematic reviews comparing antimotility agents with other anti diarrhoeals in controlling chronic diarrhoea in HIV positive individuals is also needed, weighing benefit against cost and availability. This will aid practice, especially in resource limited settings, and further improve the quality of life of patients.

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- * Indicates the major publication for the study

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CHARACTERISTICS OF STUDIES

Characteristics of included studies [ordered by study ID]

Ilboudo 1997

Methods	Randomized double blind placebo controlled. Clinical evaluation of patients for 5 consecutive days. Fluid replacement in both arms as necessary. Diarrhoea drugs were prescribed.	
Participants	91 adults aged 18 - 60 from 6 university hospitals in 5 African French speaking countries were included 45 were assigned to attapulgit and 46 assigned to receive placebo. Inclusion criteria: AIDS diagnosis by CDC criteria; diarrhoea for at least 7 days (at least 4 loose stools per day) and eligible for at least 7 days hospitalization. Exclusion criteria: Receiving other therapies which might influence the study drugs (antacids, modifiers of the transit, antisectional and antibacterial drugs). Male = 62% (56/91), Female = 38% (35/91) Mean age (years) = 34, Range = 20 - 58 Mean weight (kg) = 45, Range = 29 - 73	
Interventions	6g of Attapulgit in 30cl water or matching placebo in 3 divided doses for 5 consecutive days	
Outcomes	Stool frequency recorded as participants with loose stools less than 3 per day from days 1 - 5. Stool consistency recorded as number of stools in solid state from days 1 - 5. weight gain measured as collective increase in weight of all participants from days 1 - 5. Adverse events recorded as 5 deaths but did not indicate the treatment groups it occurred. Painful symptomatology - Number of patients complaining of pains after 5 days of treatment	
Notes	Date, duration of trial, details of randomisation, source of funding, etthics approval, other treatments given and details of adverse events were not recorded in the trial report. Efforts at getting further information from the authors were not successful	
<i>Risk of bias</i>		
Item	Authors' judgement	Description
Allocation concealment?	Unclear	B - Unclear

Characteristics of excluded studies [ordered by study ID]

Study	Reason for exclusion
Compean 1994	Did not compare an antimotility agent or an adsorbent with another antimotility agent, an adsorbent, placebo or no treatment

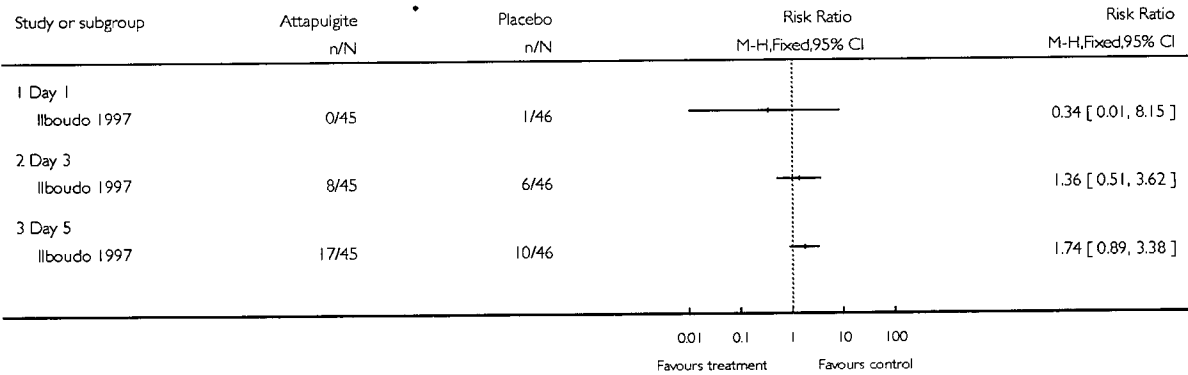
DATA AND ANALYSES

Comparison 1. Treatment with an adsorbent versus placebo

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Comparison of stool consistency with Attapulgitte versus Placebo	1		Risk Ratio (M-H, Fixed, 95% CI)	Totals not selected
1.1 Day 1	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable
1.2 Day 3	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable
1.3 Day 5	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable
2 Comparison of stool frequency with Attapulgitte versus Placebo	1		Risk Ratio (M-H, Fixed, 95% CI)	Totals not selected
2.1 Day 1	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable
2.2 Day 3	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable
2.3 Day 5	1		Risk Ratio (M-H, Fixed, 95% CI)	Not estimable

Analysis 1.1. Comparison 1 Treatment with an adsorbent versus placebo, Outcome 1 Comparison of stool consistency with Attapulgitte versus Placebo.

Review: Antimotility agents for chronic diarrhoea in people with HIV/AIDS
Comparison: 1 Treatment with an adsorbent versus placebo
Outcome: 1 Comparison of stool consistency with Attapulgitte versus Placebo

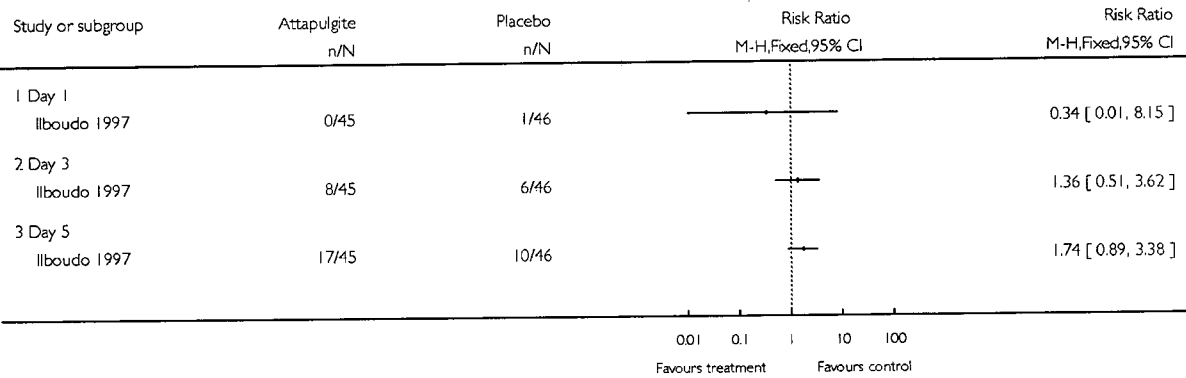


Analysis 1.2. Comparison 1 Treatment with an adsorbent versus placebo, Outcome 2 Comparison of stool frequency with Attapulgitte versus Placebo.

Review: Antimotility agents for chronic diarrhoea in people with HIV/AIDS

Comparison: 1 Treatment with an adsorbent versus placebo

Outcome: 2 Comparison of stool frequency with Attapulgitte versus Placebo



WHAT'S NEW

Last assessed as up-to-date: 13 September 2007.

Date	Event	Description
11 August 2008	Amended	Converted to new review format.

HISTORY

Protocol first published: Issue 1, 2006

Review first published: Issue 4, 2008

Date	Event	Description
14 September 2007	New citation required and conclusions have changed	Substantive amendment

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19/6

CONTRIBUTIONS OF AUTHORS

Both authors formulated the review topic and reviewed the search output, Chukwuemeka Nwachukwu developed the protocol, contacted experts and wrote the final report with inputs from Joseph Okebe.

DECLARATIONS OF INTEREST

None

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External sources

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- South African Cochrane Centre (SACC) HIV/AIDS Mentoring Programme, South Africa.

INDEX TERMS

Medical Subject Headings (MeSH)

Acquired Immunodeficiency Syndrome [complications; drug therapy]; Antidiarrheals [*therapeutic use]; Chronic Disease; Diarrhea [*drug therapy]; Gastrointestinal Transit [drug effects]; HIV Infections [*complications; drug therapy]; Immunocompromised Host; Magnesium Compounds [*therapeutic use]; Silicon Compounds [*therapeutic use]

MeSH check words

Adult; Humans

NSP4 elicits age-dependent diarrhea and Ca^{2+} -mediated I^- influx into intestinal crypts of CF mice

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Morris, Andrew P., Jason K. Scott, Judith M. Ball, Carl Q.-Y. Zeng, Wanda K. O'Neal, and Mary K. Estes. NSP4 elicits age-dependent diarrhea and Ca^{2+} -mediated I^- influx into intestinal crypts of CF mice. *Am. J. Physiol.* 277 (Gastrointest. Liver Physiol. 40): G431–G444, 1999.—Homologous disruption of the murine gene encoding the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) leads to the loss of cAMP-mediated ion transport. Mice carrying this gene defect exhibit meconium ileus at birth and gastrointestinal plugging during the neonatal period, both contributing to high rates of mortality. We investigated whether infectious mammalian rotavirus, the recently characterized rotaviral enterotoxin protein NSP4, or its active NSP4_{114–135} peptide, can overcome these gastrointestinal complications in CF (CFTR^{m3Bay} null mutation) mice. All three agents elicited diarrhea when administered to wild-type (CFTR^{+/+}), heterozygous (CFTR^{+/-}), or homozygous (CFTR^{-/-}) 7- to 14-day-old mouse pups but were ineffective when given to older mice. The diarrheal response was accompanied by non-age-dependent intracellular Ca^{2+} mobilization within both small and large intestinal crypt epithelia. Significantly, NSP4 elicited cellular I^- influx into intestinal epithelial cells from all three genotypes, whereas both carbachol and the cAMP-mobilizing agonist forskolin failed to evoke influx in the CFTR^{-/-} background. This unique plasma membrane halide permeability pathway was age dependent, being observed only in mouse pup crypts, and was abolished by either the removal of bath Ca^{2+} or the transport inhibitor DIDS. These findings indicate that NSP4 or its active peptide may induce diarrhea in neonatal mice through the activation of an age- and Ca^{2+} -dependent plasma membrane anion permeability distinct from CFTR. Furthermore, these results highlight the potential for developing synthetic analogs of NSP4_{114–135} to counteract chronic constipation/obstructive bowel syndrome in CF patients.

cystic fibrosis transmembrane conductance regulator; halide permeability; NSP4 enterotoxin; rotavirus

THE MOST COMMONLY REPORTED gastrointestinal manifestation in cystic fibrosis (CF) is malabsorption due to pancreatic insufficiency, caused by occlusion of the pancreatic ducts by abnormally viscous mucus secretions. This alteration in intraluminal fluid and mucus content extends to other solid organs and hollow viscera of the gut, creating the clinical conditions of meconium ileus at birth and distal intestinal obstruc-

tion syndrome, chronic constipation with acquired megacolon, and rectal prolapse in older individuals (1).

Underlying these gastrointestinal complications is the basic defect in CF, which is caused by mutations in the gene that encodes the CF transmembrane conductance regulator (CFTR; Ref. 43). The most commonly reported mutation in CF, accounting for the genetic defect in 60–70% of the chromosomes of CF individuals, is a three-base-pair deletion that removes phenylalanine at position 508 (ΔF508) of the CFTR protein (54). CF patients homozygous for this mutation exhibit the severe clinical phenotype of lung disease, pancreatic insufficiency, and predisposition to gastrointestinal obstruction (48). Electrophysiological analysis of the gut mucosa isolated from these individuals demonstrates a lack of cAMP-dependent fluid secretion (6, 37, 53). Reduced fluid transport, which is the primary cellular defect of CF epithelia, is believed to significantly enhance the pathology of mucus plugging in the gut.

Over the past six years, mouse models for CF that duplicate the pathophysiological phenotype of CF in the human intestine have been produced with the use of gene-targeting techniques. Transgenic CFTR-deficient animals fail to exhibit CFTR-mediated fluid secretion and present with gastrointestinal disease (15). In this study, we utilized the CFTR^{m3Bay} null mutation mouse as a model for CF to investigate whether infectious rotavirus or the novel rotaviral enterotoxin NSP4 (3) can cause diarrhea. These mice fail to express functional CFTR protein because of multiple stop codons engineered within exon 3 of the murine CFTR genome (23).

Rotaviruses are the leading cause of severe gastroenteritis in infants and young animals (25). We have recently shown that NSP4, a rotavirus nonstructural protein (16), can cause diarrhea in young mice (3). Furthermore, electrophysiological analyses of intact intestinal mucosa from mice revealed that NSP4 mobilizes Ca^{2+} to mimic the secretory effects of the cholinergic agonist carbachol (CCh) in potentiating cAMP-dependent fluid secretion (3). We therefore asked whether NSP4 could evoke a diarrheal response in CFTR-depleted mice that exhibit no functional cAMP-dependent secretory pathway. Here, we demonstrate that NSP4 injected intraperitoneally into CFTR-deficient (CFTR^{-/-}) mouse pups induces an age-dependent diarrhea. Hence, CFTR-mediated changes in intestinal fluid transport were not directly involved in NSP4-elicited diarrhea. However, NSP4 was found

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to mobilize Ca^{2+} in the crypt epithelia of both small and large intestine from wild-type ($\text{CFTR}^{+/+}$) as well as $\text{CFTR}^{-/-}$ pup and adult mice. These ex vivo data correlated with our in vitro findings in human gastrointestinal cell lines, in which NSP4 was shown to mobilize intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) via phospholipase C (PLC) activation and inositol 1,4,5-trisphosphate (IP_3) production (13). To investigate in greater detail the relationship between NSP4-induced epithelial cell Ca^{2+} mobilization and our previously reported age-dependent effects of NSP4 on murine intestinal mucosa Cl^- secretory current generation (3), we measured plasma membrane halide permeability changes ex vivo within the epithelial cells of isolated distal colon crypts. We found that NSP4 elicited age-dependent I^- influx into mouse pup crypts, inhibited by either the removal of bath Ca^{2+} or the transport inhibitor DIDS. The fact that this Ca^{2+} -dependent plasma membrane halide permeability pathway was not CFTR may provide a possible explanation as to why $\text{CFTR}^{-/-}$ mouse pups exposed to NSP4 develop age-dependent diarrhea.

MATERIALS AND METHODS

Reagents. Fura 2-AM and 6-methoxy-*N*-(3-sulfo-propyl) quinolinium (SPQ) were purchased from Molecular Probes (Eugene, OR). All other reagents, including DIDS, were purchased from Sigma (St. Louis, MO). NSP4 protein was fast-performance liquid chromatography- and affinity-purified from Sf9 insect cells infected with a recombinant baculovirus pAC461-G10 expressing simian rotavirus gene 10 (encoding NSP4), as described previously (13). The NSP4₁₁₄₋₁₃₅ peptide was synthesized at the University of Pittsburgh protein core laboratory and characterized as previously described (3).

Experimentally induced rotaviral infection or administration of NSP4 protein and NSP4₁₁₄₋₁₃₅ peptide to mouse pups. The simian rotavirus strain SA11 clone 3 (18) was used to infect neonatal CD-1 or $\text{CFTR}^{-/-}$ (23) mouse pups between the ages of 6 and 8 days. The genotype of the $\text{CFTR}^{-/-}$ mice was determined by polyacrylamide gel analysis of PCR products generated from DNA extracted from the tails of 1- to 2-day-old mice with the use of primers, as described previously (23). Either 10 or 20 diarrheal dose 50 of SA11 was administered in 50 μl of medium 199 by intragastric gavage. Alternatively, purified NSP4 protein or NSP4₁₁₄₋₁₃₅ was administered to C57B $\text{CFTR}^{-/-}/\text{CFTR}^{+/+}$ or CD-1 $\text{CFTR}^{+/+}$ mouse pups in a final volume of 50 μl of PBS by intraperitoneal injection. The doses used were 0.5 nmol of NSP4 protein or 100 nmol of NSP4₁₁₄₋₁₃₅ peptide because these doses were shown to be effective at inducing diarrhea in CD-1 $\text{CFTR}^{+/+}$ mouse pups (3).

Diarrheal activity measurements. To determine the presence of diarrhea following protein or peptide treatment, each mouse pup was examined every 1–2 h for the first 8 h and at 24 h after inoculation by gently pressing the abdomen. After virus inoculation, mouse pups were monitored twice a day for 4 days. Diarrhea was noted and scored from 1 to 4, with a score of 1 reflecting loose yellow stool and a score of 4 indicating completely liquid stool. A score of 2 (mucous with liquid stool, some loose but solid stool) and above was considered diarrhea. The scoring was performed on coded animals by a single person.

Crypt isolation and dye loading. Epithelial crypts from $\text{CFTR}^{-/-}$ mice (aged 8–12 days) were isolated from 3-cm segments of the mid to distal small intestine, 6 cm proximal to

Bauhin's valve, and from the distal colon. After euthanasia by an overdose of ether and cervical dislocation, the entire small intestine and colon from the mouse were removed and flushed with ice-cold physiological saline. Individual intestinal segments were then mounted onto Perspex paddles and were immersed in Ca^{2+} -free standard Krebs buffered saline (in mmol/l: 107 NaCl, 4.5 KCl, 0.2 NaH_2PO_4 , 1.8 Na_2HPO_4 , 10 glucose, and 10 EDTA), and continuously gassed with 5% CO_2 -95% O_2 at 37°C for 10–20 min. The crypts were then separated from the overlying mucosa by mechanical vibration for 30 s into ice-cold KCl HEPES saline (in mmol/l: 100 potassium gluconate, 20 NaCl, 1.25 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, 5 sodium pyruvate, and 0.1% BSA, pH 7.4), resembling the intracellular medium. Suspended crypts were then deposited (1,200 rpm for 1 min) onto poly-L-lysine-coated microscope coverslips (0 oz) with the use of a Shandon Cytospin cell preparation system (13). The coverslips with crypts were then attached with vacuum grease to the base of customized perfusion wells and loaded in the dark with either Ca^{2+} -sensing or Cl^- -sensing dye.

Fura 2-AM loading. Isolated crypts were incubated with 10 μM fura 2-AM at room temperature for 15–20 min. The coverslips were then mounted on an inverted Nikon microscope and superfused with standard HEPES-buffered extracellular saline (in mmol/l: 140 NaCl, 4.7 KCl, 1.13 MgCl_2 , 10 HEPES, 10 glucose, and 1 CaCl_2 , pH 7.4) for 5 min before imaging.

SPQ loading. Isolated crypts were loaded with SPQ at room temperature by a 6-min exposure to hyposmotic (80 mosM Cl^- or NO_3^-) HEPES-buffered extracellular saline containing 5 mM SPQ. After loading, the crypts were allowed to recover in isosmotic (140 mosM Cl^- or NO_3^-) HEPES-buffered extracellular saline for 10–15 min. This period was used when performing anion channel inhibitor studies to preload the crypts so that the reported spectral interference of 0.5 mM DIDS with SPQ fluorescence was minimized (61). DIDS binds irreversibly to cellular membranes; therefore, crypts were loaded and the bathing saline was changed before experimentation. The solution temperature was maintained at 37°C throughout these studies by prewarming the extracellular solutions and by water-jacketing the oil-immersion lens of the inverted microscope.

NSP4/NSP4₁₁₄₋₁₃₅ addition to isolated crypts. Small (100 μl) volumes of either the NSP4 protein or NSP4₁₁₄₋₁₃₅ peptide were superfused onto the isolated crypts during regular bath flow by N_2 pressure injection with the use of a Picospritzer. Low-resistance glass pipettes were filled with either compound dissolved in HEPES-buffered extracellular saline, and the tip was maneuvered close to the isolated crypt. Peptide or protein was released directly into the vicinity of the crypt for 40 s before being washed away by the bath flow.

$[\text{Ca}^{2+}]_i$ imaging. All experiments were carried out with the aid of a high-resolution camera imaging system as described previously (13). In brief, light emitted from fura 2-loaded cells at 510 nm was captured by an intensified video camera after exposure to both 340- and 380-nm excitation light. The camera signal was then digitally encoded and processed with image analysis software (IMAGE1/FL, Universal Imaging, Media, PA). The background-subtracted images were ratioed on a pixel-by-pixel basis to yield a bitmap field. Calibration of the fura 2 dye fluorescence was carried out with the use of the ionophore ionomycin under Ca^{2+} -free and Ca^{2+} -saturating conditions as described previously, and the $[\text{Ca}^{2+}]_i$ was calculated according to the Grynkiewicz equation (21). The $[\text{Ca}^{2+}]_i$ values of individual field pixels obtained by this procedure were color coded and displayed on an RGB monitor before being stored on the hard disk. Six collection areas were

chosen along the longitudinal axis (base to neck) of the crypt for spatial and time-dependent analysis of $[\text{Ca}^{2+}]_i$. The averaged ratio signal obtained from each of the six areas was digitally saved as a log file. The collected values from the six areas imaged within a single experiment were averaged together to give an experimental observation of one ($n = 1$). Values obtained from similarly placed collection areas along the longitudinal axis of different crypts were also averaged to see if any spatial localization to the Ca^{2+} signal existed. All protocols were completed within 1 h after crypt isolation.

Intracellular I^- imaging. The same high-resolution camera imaging system described above was utilized for these studies. Excitation for SPQ fluorescence was provided by a barrier filter centered at 365 ± 10 nm, reflected into the microscope objective by a 400-nm dichroic mirror. A neutral density filter (1.5 OD) was included in the light path to minimize photobleaching. The fluorescence emission from the SPQ-loaded cells passed through a 450 ± 25 -nm barrier filter before being detected by the intensifier/camera. Fluorescent images were acquired at 0.8-s intervals and averaged over eight frames to yield a 540×480 field of mean pixel intensities. Six collection areas were then chosen within the longitudinal axis (base to midregions) of the crypt for spatial and time-dependent analysis of cellular SPQ fluorescence. SPQ does not exhibit excitation or emission shifts on anion interaction. Thus ratiometric imaging is not possible, and dye fluorescence is affected by volume changes within the cell (58). Both cAMP- and Ca^{2+} -mobilizing agonists have been reported to induce shrinkage without compensatory volume recovery in the lower to midregion of rat distal colonic crypts over a 5- to 30-min time course (11). To determine the extent of this effect over the time period of SPQ quenching (1–3 min), colonic crypts isolated from the distal colon of the mouse were loaded with the AM form of calcein, a dye inert to Ca^{2+} and pH changes in cells within the physiological range (8, 9). Cell volume measured over the first 30 s of agonist addition to the bath did not change appreciably ($<2\%$) within the lower/midcrypt region (data not shown). These regions were utilized for all studies. Furthermore, to minimize possible volume effects on dye fluorescence, we chose an experimental design in which SPQ fluorescence quench by halide influx was employed rather than dequenching following halide efflux. This ensured that agonist-induced cell shrinkage, if present, would act to decrease rather than to potentiate changes in plasma membrane halide permeability. Thus our reported values of SPQ quench may modestly underestimate agonist-evoked halide influx. The collected values from the six areas imaged within a single experiment were averaged together to give an experimental observation of one ($n = 1$).

Calculation of Stern-Volmer constants for halide quenching of SPQ. SPQ fluorescence is quenched collisionally by halide anions with different potency. Nonphysiological anions such as thiocyanate (SCN^-), Br^- , F^- , and I^- quench strongly in free solution with an efficacy greater than Cl^- ($\text{Cl}^- < \text{Br}^- < \text{SCN}^- < \text{I}^-$), whereas NO_3^- does not quench at all (27). The Stern-Volmer equation that describes this interaction is $F_0/F_{\text{anion}} = 1 + K_q[\text{anion}]$, where F_0 is the fluorescence in the absence of anion, F_{anion} is the fluorescence in the presence of a given anion concentration, K_q (slope) is the Stern-Volmer quenching constant (in M^{-1}), and $[\text{anion}]$ is the concentration of halide utilized (27). Stern-Volmer quenching constants for Cl^- , Br^- , and I^- anions were calculated from linear curves obtained over a concentration range of 0–150 mM halide (see RESULTS). The near-linear Stern-Volmer quench curve for I^- (Fig. 1) and the fact that plasma membrane I^- permeability changes occur largely through conductive pathways (see

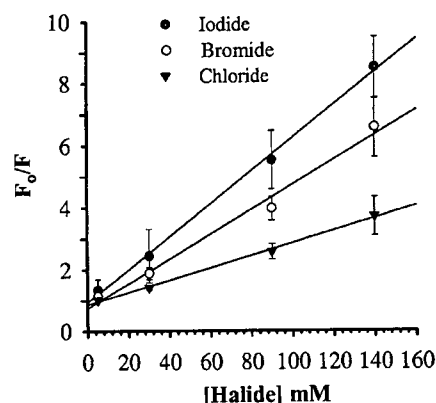


Fig. 1. Stern-Volmer plot for quenching of 6-methoxy-*N*-(3-sulfo-propyl)quinolinium (SPQ) fluorescence by Cl^- , Br^- , and I^- . Isolated crypts from distal colon of wild-type cystic fibrosis transmembrane conductance regulator ($\text{CFTR}^{+/+}$) and heterogeneous ($\text{CFTR}^{+/-}$) mice were loaded with SPQ. Intracellular SPQ quench by different intracellular halide concentrations was induced with dual ionophore technique. F_0 and F represent background and dye leak subtracted values of fluorescence in absence and in presence of quenching halide, respectively. Values are means $\pm$ SD.

DISCUSSION and Refs. 36 and 63) led us to choose this anion to quantify agonist effects in isolated crypts.

To calibrate SPQ fluorescence in cells, the double ionophore technique, with the use of high- K^+ solutions containing nigericin (5 μM) and tributyltin (10 μM), was employed (7). In these experiments, isolated crypts were loaded with SPQ and hyposmotic NO_3^- (80 mM KNO_3) potassium saline and then were transferred to isosmotic NO_3^- (140 mM KNO_3) potassium saline containing the above ionophores. F_0/F_{anion} values were obtained by perfusing the crypts with varying concentrations of test halide (Cl^- , Br^- , or I^-) plus ionophore followed by SCN^- in the presence of valinomycin (5 μM) to completely quench the intracellular SPQ signal and obtain basal fluorescence values. Dye leakage was $\sim 17\%$ of the total cellular signal over 60 min (0.0125 ± 0.008 fluorescent units/s, $n = 60$). F_0 and F_{anion} values were corrected for this signal on a per-experiment basis by interpolation from the fluorescence intensity, measured in cells bathed in Cl^- -free medium at the onset of the experiment and at the end of the experiment or by back extrapolation from the rate of change measured during the SCN^- fluorescence minimal quench signal (i.e., $F_0 - F_{\text{ex}}$ and $F_{\text{anion}} - F_{\text{ex}}$, where F_{ex} = background fluorescence at the time of measurement). Both methods gave comparable values. All experimental protocols were completed within 15–20 min. The Stern-Volmer quench constants for halide quenching of SPQ in pup and adult crypts (see RESULTS) were utilized to calculate the halide influx rates in the standard experimental procedure according to the following equation: $J_{\text{I}^-} = (F_0/K_q \cdot F^2) \cdot (\Delta F/\Delta t)$, in which $\Delta F/\Delta t$ is the rate of fluorescence change measured at time 0. In addition to this method, running averages of halide influx at 1.6-s intervals during the first 40 s of the fluorescence quench were calculated.

Standard experimental procedure for measuring agonist-stimulated I^- influx. SPQ-loaded crypts were perfused with HEPES-buffered NaNO_3 containing extracellular saline, and, after establishment of a stable fluorescence signal, bath NO_3^- was replaced with I^- (140 mosM). The quenched SPQ fluorescent signal (NO_3^- does not quench SPQ nor compete with I^-) was recorded over predefined measurement windows along the base to midcrypt axis. Basal rates of I^- influx (J_{I^-}) were then calculated with the use of the Stern-Volmer quench constants for I^- obtained for either mouse pup or adult crypts

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(see *Calculation of Stern-Volmer constants for halide quenching of SPQ and RESULTS*). Cl⁻ was then reintroduced, and I⁻ was simultaneously removed from the bath. A single agonist was administered during this recovery phase (forskolin, FSK) or after establishment of a new stable fluorescence signal (CCh or NSP4) when bath I⁻ was reintroduced in Cl⁻-free saline. The resulting agonist-stimulated SPQ quench curve was recorded, and J_{I-agonist} was calculated. Agonist-induced changes in I⁻ influx rate were then calculated from pooled values of J_{I-agonist} and J_{I-basal} as ΔJ_I = (J_{I-agonist} - J_{I-basal}). In addition to this approach, mean ± SD rates of fluorescence change at 1.6-s intervals over the first 40 s of fluorescence quench were graphically displayed in histogram format as change in agonist-induced quench rate, ΔR_I = (ΔF/Δt_{agonist} - ΔF/Δt_{basal}). This later approach allowed us to determine latency and to record changes in dye quench unrelated to initial (*time 0*) changes in quench rate. All SPQ-loaded crypts were superfused with saline at the same bath perfusion rate (4 ml/min); solution exchange in the experimental chamber (200 μl) occurred within 3 s.

Statistical analysis. To determine statistical significance of differences between observations within an experiment, the paired Student's *t*-test was used. For statistical differences between experiments, the unpaired Student's *t*-test was used. Between 6 and 12 separate experimental observations pooled from different dye loadings were routinely collected for each experimental condition. The Fisher's exact test was used to estimate the probability of difference for diarrheal activity measurements within CF and non-CF groups in which the tabulated frequency of occurrence was too small for χ² analysis.

RESULTS

Effect of orally administered rotavirus and injected viral NSP4 peptide(s) in mouse pups. Inoculation of CFTR^{-/-} mice with infectious SA11c13 rotavirus caused diarrhea in four of five mice within 24–48 h (Table 1). When CFTR^{+/+} or CFTR^{+/-} mouse pups from the same C57B1/J6 background were infected with the SA11 virus, ~78% of the mice exhibited diarrhea (Table 1). Intraperitoneal injection of intact NSP4 protein or NSP4₁₁₄₋₁₃₅ was similarly found to elicit diarrhea in CFTR^{+/+} or CFTR^{+/-} mouse pups (66 and 70% respond-

ing, respectively). CFTR^{-/-} mouse pups inoculated with these reagents exhibited diarrhea in 50% and 43% of these mice, respectively (Table 1). Diarrheal content was graded as equally severe as that encountered in CFTR^{+/+} mice (3). Although the incidence of diarrhea caused by NSP4 protein or NSP4₁₁₄₋₁₃₅ appeared lower in the CF mouse studies, there was no significant difference between CFTR^{+/+}, CFTR^{+/-}, and CFTR^{-/-} littermates (Fisher's exact test for 2 × 2 contingency tables; Ref. 32). All three reagents (SA11 virus, NSP4 protein, or NSP4₁₁₄₋₁₃₅) were effective diarrheal agents. In contrast, when infectious SA11c13 virus was given to either adult CFTR^{+/+}, CFTR^{+/-}, or CFTR^{-/-} mice, none exhibited diarrhea (Table 1).

NSP4 peptide mobilizes [Ca²⁺]_i levels in epithelial cells isolated from the small and large bowel of both CFTR^{+/+} and CFTR^{-/-} mice. Rotaviral protein production has been correlated with changes in [Ca²⁺]_i homeostasis (39) and with Ca²⁺-dependent cytotoxicity during viral infection in cultured cells (33, 34). With the use of the human colonic epithelial cell line HT-29 clone 19A, which expresses plasmalemmal proteins found in both small and large intestinal cells, we recently demonstrated that exogenously added NSP4 and NSP4₁₁₄₋₁₃₅ peptide causes [Ca²⁺]_i mobilization by PLC-mediated IP₃ production (13). We therefore investigated whether the diarrheal effects of NSP4 and NSP4₁₁₄₋₁₃₅ peptide in vivo were also associated with native mucosal intestinal cell [Ca²⁺]_i mobilization. Fluorescence video microscopy was used to study the effects of exogenous NSP4 addition on [Ca²⁺]_i in intestinal crypts isolated from either small or large bowel of 8- to 13-day-old mouse pups.

Small intestinal crypts. Crypts from the small intestine were chosen in preference to villi, which dissociated during isolation. Addition of 100 nM NSP4 protein, a dose shown to promote near-maximal [Ca²⁺]_i rises in vitro (13), elicited rapid rises in [Ca²⁺]_i that lasted 2–5 min in CFTR^{+/+}, CFTR^{+/-}, and CFTR^{-/-} small intestinal crypts. Resting [Ca²⁺]_i values of 126 ± 28 nM (CFTR^{-/-}, *n* = 13) and 110 ± 63 nM (CFTR^{+/+} and CFTR^{+/-}, *n* = 14) increased to peak values of 205 ± 54 nM and 260 ± 116 nM, respectively (Table 2). A representative example (Fig. 2, A–C) shows consecutive images taken before, during, and after NSP4 addition to an epithelial crypt isolated from a CFTR^{-/-} mouse. No significant difference was seen between mouse CFTR genotypes with respect to basal, peak, or net Ca²⁺ values (*P* > 0.01, Student's *t*-test; Table 2). These results indicated that NSP4 was an effective Ca²⁺-mobilizing agonist in the small intestine and that Ca²⁺ homeostasis, both basal and agonist-stimulated, is unaffected by the CFTR gene knockout. Not all experimental crypts responded; 27 of 108 (25%) gave [Ca²⁺]_i rises. Increasing the dose of NSP4 from 100 to 500 nM and above did not increase the number of NSP4-responsive crypts (3 of 18 responded = 17%; data not shown).

Colonic crypts. When isolated crypts from the distal colon of CFTR^{+/+}, CFTR^{+/-}, and CFTR^{-/-} mice were superfused with 100 nM NSP4 protein, [Ca²⁺]_i rises

Table 1. Induction of diarrhea by rotavirus infection or NSP4 in CFTR^{-/-} mice

Inoculum	Dose	Route	Mouse Genotype		P ^{FET}
			CFTR ^{+/+} or CFTR ^{+/-}	CFTR ^{-/-}	
Mice 6–8 days old					
Rotavirus (SA11)	10–20 DD ₅₀	Oral	7/9 (78%)	4/5 (80%)	>0.05
NSP4 protein	0.5–2 nmol	ip	8/12 (66%)	3/6 (50%)	>0.05
NSP4 ₁₁₄₋₁₃₅	100 nmol	ip	7/10 (70%)	3/7 (43%)	>0.05
Mice >21 days old					
Rotavirus (SA11)	10–20 DD ₅₀	Oral	0/3 (0%)	0/5 (0%)	

Values are number of responders/total number of mice tested. Mice were genotyped after birth. NSP4 protein was expressed and purified as previously described (10). CFTR, cystic fibrosis transmembrane conductance regulator; DD₅₀, diarrhea dose 50; P^{FET}, Fisher's exact test.

Table 2. Summary of the effects of 100 nM NSP4 and 100 μM CCh on $[\text{Ca}^{2+}]_i$ levels in either $\text{CFTR}^{-/-}$ or $\text{CFTR}^{+/+, +/-}$ small and large intestine mouse crypts

Tissue	Genotype	CCh				NSP4			
		Basal $[\text{Ca}^{2+}]_i$	Peak $[\text{Ca}^{2+}]_i$	$\Delta[\text{Ca}^{2+}]_i$	n	Basal $[\text{Ca}^{2+}]_i$	Peak $[\text{Ca}^{2+}]_i$	$\Delta[\text{Ca}^{2+}]_i$	n
Colon	$\text{CFTR}^{-/-}$	98 $\pm$ 12	163 $\pm$ 16	65 $\pm$ 14*	6	113 $\pm$ 24	211 $\pm$ 64	97 $\pm$ 52	18
	$\text{CFTR}^{+/-, +/+}$	89 $\pm$ 46	335 $\pm$ 155	255 $\pm$ 155*	16	88 $\pm$ 35	242 $\pm$ 105	151 $\pm$ 84	36
Ileum	$\text{CFTR}^{-/-}$	142 $\pm$ 36	219 $\pm$ 55	77 $\pm$ 38	13	126 $\pm$ 28	205 $\pm$ 54	80 $\pm$ 37	13
	$\text{CFTR}^{+/-, +/+}$	95 $\pm$ 55	203 $\pm$ 104	106 $\pm$ 64	12	110 $\pm$ 63	260 $\pm$ 116	160 $\pm$ 93	14

Values are mean intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) values (in nM) $\pm$ SD recorded from responding crypts. Experiments were performed on 8- to 13-day-old mice. Only 25% of crypt preparations responded. CCh, carbachol. * $P < 0.008$. All other comparisons between groups were not significant.

similar to those obtained from small intestinal crypts were recorded ($P > 0.1$, Student's t -test; Table 2). Peak NSP4-induced $[\text{Ca}^{2+}]_i$ values were similar in all three genetic backgrounds, although basal values were slightly, but not significantly, higher in $\text{CFTR}^{-/-}$ mice when all samples were pooled ($P > 0.01$, Student's t -test; Table 2). In our in vitro studies, we found that the NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization was susceptible to predepletion by other agonists (remaining unresponsive for tens of minutes; Ref. 13). In contrast, NSP4-induced $[\text{Ca}^{2+}]_i$ release in both ex vivo colonic and ileal mucosal cell preparations was not desensitized by repetitive agonist administration ($n = 15$). In the $\text{CFTR}^{-/-}$ mouse crypt preparation shown in Fig. 3, identical $[\text{Ca}^{2+}]_i$ rises were seen for consecutive NSP4 or CCh followed by NSP4 challenge. Ca^{2+} homeostasis in the isolated crypt therefore appears to be more tightly regulated than in colonic cell lines.

To address why the NSP4 response rate of both small and large intestinal crypts was not $>25\%$, we quantified the responsiveness of isolated crypts to the cholinergic agonist CCh. The addition of 100 μM CCh to the bath elicited $[\text{Ca}^{2+}]_i$ rises in a similar percentage of isolated crypts from both small and large intestine (33% responded; Table 2). Lower, more physiological doses of CCh would likely produce identical success rates for both agonists. These findings suggested that a more general cellular phenomenon, other than a lack of

NSP4 receptors, was accountable for this effect. Because both M_3 -muscarinic receptors and the cellular response to NSP4 have been shown to be sensitive to protease digestion (13), true in vivo NSP4 potency may have been masked.

Spatial analysis of the Ca^{2+} -mobilizing effects of NSP4 revealed no regional differences along the longitudinal axis of either colonic or small intestinal crypts. Mean resting values for base, mid, and neck regions (separated by $\sim 25 \mu\text{m}$) were 112 ± 142 , 100 ± 25 , and 102 ± 39 nM, rising to 250 ± 99 , 229 ± 97 , and 215 ± 79 nM, respectively ($n = 29$). The lack of localization-dependent effects on $[\text{Ca}^{2+}]_i$ indicates that, regardless of the cell maturity, the membranes of epithelial cells within the crypt remained responsive to NSP4. An example of this spatial homogeneity is shown in Fig. 4. In Fig. 4, a sustained component to NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization is observed that disappeared on removal of NSP4 from the bath. Nominally Ca^{2+} -free conditions did not affect the Ca^{2+} response to NSP4 protein ($n = 12$, data not shown), confirming that the major source of the Ca^{2+} rise was from intracellular stores. However, under extracellular Ca^{2+} -free conditions, no sustained component was observed, indicating that Ca^{2+} influx also occurred. The effects of NSP4 ex vivo reproduced those recorded in vitro, which have been linked to the intracellular production of IP_3 (13).

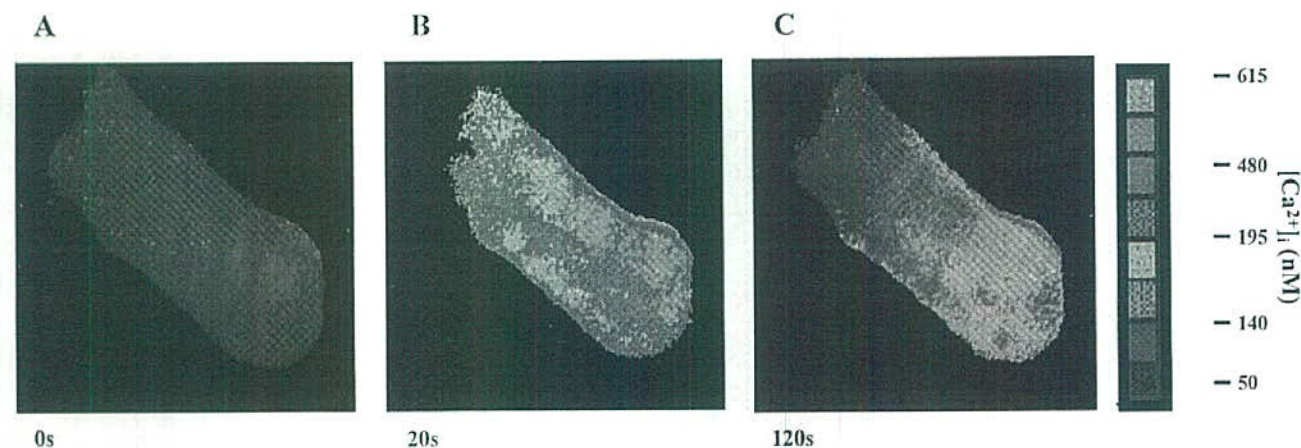


Fig. 2. Consecutive pseudocolor images of retroviral enterotoxin protein NSP4-induced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) rise in an isolated $\text{CFTR}^{-/-}$ small intestinal mouse crypt, loaded with fura 2. A: resting Ca^{2+} level of ~ 120 nM before agonist stimulation. B: peak Ca^{2+} level approaching 220 nM immediately after addition of 100 nM NSP4. C: gradual return of crypt to resting Ca^{2+} levels ~ 1.5 min after addition of NSP4.

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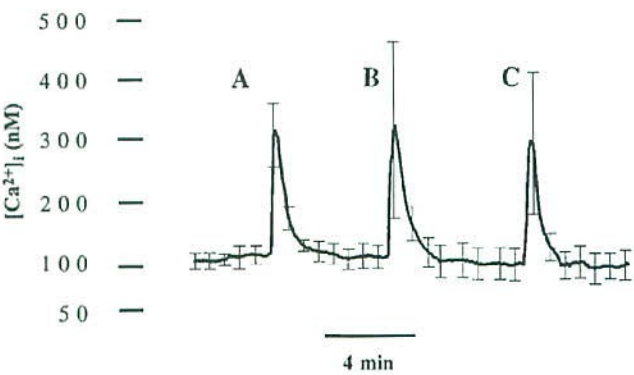


Fig. 3. A representative example of a single crypt. Average $[Ca^{2+}]_i$ values from 6 measurement areas along length of an isolated CFTR $^{-/-}$ colonic mouse crypt, showing effect of 100 nM NSP4 (A), 500 μ M carbachol (CCh, B), and 100 nM NSP4 (C). SD values are shown as error bars.

NSP4 peptide mobilizes $[Ca^{2+}]_i$ levels in crypt epithelial cells from both pup and adult mice. Isolated colonic crypts from either normal (CFTR $^{+/+}$) C57B or BALB/c mice were loaded with fura 2 and exposed to 100 nM NSP4. In the example shown (BALB/c, Table 3), NSP4 mobilized Ca^{2+} in crypts from both 7- to 12-day- and >25-day-old mice. Although the mean number of BALB/c crypt responses was higher than that observed in the C57B background (data not shown), there was no age dependency to the magnitude of the NSP4-induced $[Ca^{2+}]_i$ rise (values were not statistically significant, unpaired t -test, $P > 0.05$, $n = 13$; Table 3). When CCh (100 μ M) was applied after a NSP4-induced Ca^{2+} mobilization, 75% of pup crypts and 80% of adult crypts responded with a second $[Ca^{2+}]_i$ rise ($n = 13$). In this instance, the net agonist-induced $[Ca^{2+}]_i$ rise was significantly smaller in adult crypts ($P < 0.001$, unpaired t -test; Table 3). When CCh alone was applied to crypts,

Table 3. Summary of the effects of 100 nM NSP4 and 100 μ M CCh on $[Ca^{2+}]_i$ levels in pup and adult BALB/c mouse colonic crypts

Agonist	$\Delta[Ca^{2+}]_i$, mM			
	BALB/c pup (7–12 days old)	<i>n</i>	BALB/c adult (>25 days old)	<i>n</i>
NSP4	112 $\pm$ 55	4	64 $\pm$ 36	9
NSP4 + CCh	502 $\pm$ 170*	4	102 $\pm$ 68*	9
CCh alone	323 $\pm$ 185	21	96 $\pm$ 99	40

Values are mean $[Ca^{2+}]_i$ values $\pm$ SD recorded from responding crypts. Number of crypts responding to NSP4 was similar in both pup (19%) and adult (22%) crypts. CCh was applied to NSP4-responding crypts. When CCh was applied alone to the bath, 40% of both pup and adult crypts responded. * $P > 0.001$, Student's unpaired t -test.

a similar age-dependent trend in Ca^{2+} mobilization was observed, which failed to be significant because of the great variability in the magnitude of the peak $[Ca^{2+}]_i$ values ($P > 0.05$; Table 3). These results indicated that events distal to NSP4-elicited $[Ca^{2+}]_i$ mobilization must regulate the age- and Ca^{2+} -dependent Cl^- secretory activity of NSP4 observed in the ex vivo mouse intestine (3).

Calculation of Stern-Volmer constants for Cl^- , Br^- , and I^- in adult and pup mouse crypts. To separate carrier-mediated anion transport events from conductive anion transport in SPQ-loaded crypts, the halide I^- was used as a surrogate anion for Cl^- . I^- is not a substrate for either the Na^+ - K^+ -2 Cl^- cotransporter or Cl^- /HCO $^-$ exchanger (36, 63). This choice was not, however, without technical drawbacks, because the Stern-Volmer relationships for Br^- , I^- , and SCN^- in free solution are reported to become positively nonlinear at >15 mM halide (24). Nonlinear increases in K_q above this concentration suggest that, in addition to

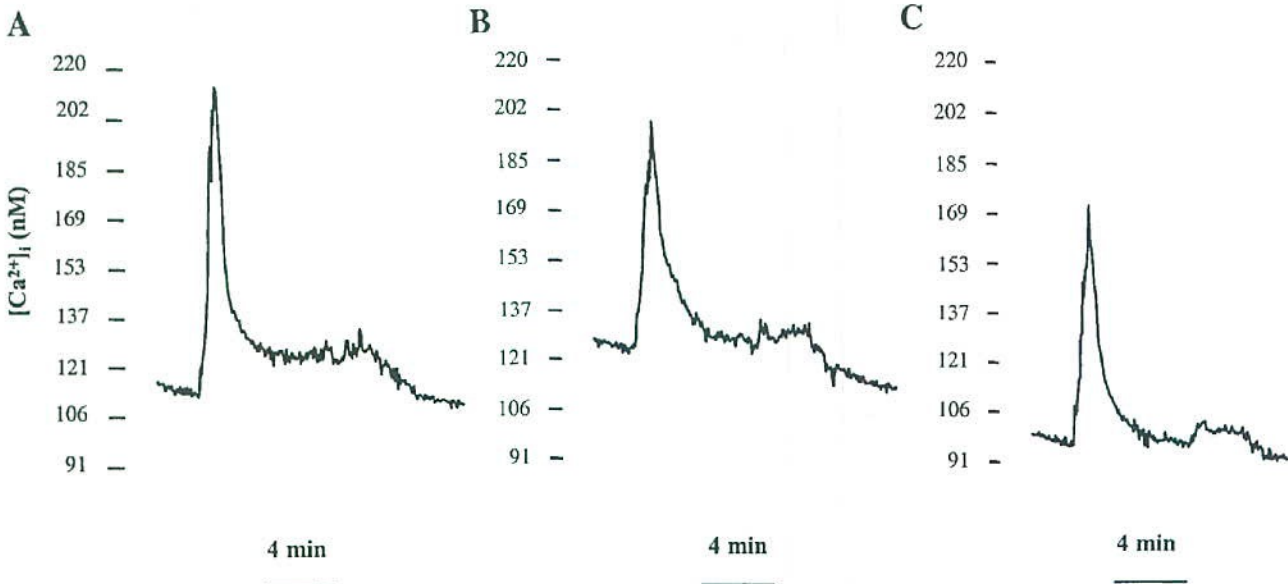


Fig. 4. $[Ca^{2+}]_i$ measurements taken from 3 different regions separated by $\sim 15 \mu$ m along longitudinal axis of a CFTR $^{-/-}$ small intestinal crypt, showing magnitude of NSP4 $[Ca^{2+}]_i$ rise at crypt base (A), midregion of crypt (B), and near neck of crypt (C). Agonist (100 nM NSP4) was washed out of bath ~ 10 min after addition.

dynamic collision interactions, static interactions occur (i.e., those leading to nonfluorescent halide-SPQ complex formation; Ref. 28). Irrespective of the exact nature of this quenching (assumed to be static buffering), the dye would become noncalibrated at high halide concentrations, compromising I^- usefulness as a surrogate anion for conductive Cl^- influx. The Stern-Volmer relationship for Cl^- on the other hand has been demonstrated to remain linear when measured both in free solution and cells and shown to be the product of the dynamic collision-quench rate constant K_q (24). Stern-Volmer relationships for Cl^- , I^- , and Br^- were constructed in SPQ-loaded crypts with the use of the dual ionophore technique (Ref. 7; see MATERIALS AND METHODS). We found that apparent Stern-Volmer quench constants for both Br^- and I^- , calculated from halide concentrations of either $<15 \text{ mM}$ or $<150 \text{ mM}$, exhibited modest $<15\%$ and slight $<5\%$ nonlinear increases in slope, respectively. This deviation was not large enough to accurately determine an additional buffering coefficient (28). Thus K_q values for Br^- and I^- were estimated by linear curve fitting and found to be 38.5 M^{-1} and 51.4 M^{-1} , respectively.¹ The K_q value for Cl^- was found to be 21.5 M^{-1} (measurements made in both pup and adult crypts, $n = 8$ per halide; Fig. 1), reflecting very well the ratio of SPQ collisional quench ($1:1.7:1.9$) reported for colonocytes (45). These values were slightly lower than those calculated in the rabbit crypts (45) and higher than those estimated in a variety of cancer cell lines (which range between 12 M^{-2} and 18 M^{-2} depending on cell type; Ref. 58). Because we failed to measure changes in tubular geometry (shrinkage) associated with agonist stimulation when confining our measurements to basal crypt areas, we did not resort to more theoretical measurements of the Stern-Volmer constant (22). With the use of these values, 50% of cellular SPQ quench would be achieved at 26, 19.6, and 47 mM Br^- , I^- , and Cl^- , respectively.

Calculation of basal and agonist-stimulated halide permeability. The dynamic K_q values determined above were used to calculate influx rates for the respective anions that were dependent on plasma membrane permeability and not on alterations in SPQ quench rate. Basal influx was in the order $\text{I}^- > \text{Cl}^- > \text{Br}^-$ (Table 4) for $\text{CFTR}^{+/+}$, $\text{CFTR}^{+/-}$, and $\text{CFTR}^{-/-}$ pup crypts. An age-dependent but not significant ($P > 0.05$) trend in basal influx was seen, with adult values being somewhat higher than those in pups. The lack of CFTR was associated with a statistically significant ($P < 0.001$) decrease in basal halide influx rate (J_{halide}) in $\text{CFTR}^{-/-}$ mice (Table 4).

¹ When utilizing I^- and NO_3^- -containing saline, it could be argued that highly membrane-permeable oxidation species (e.g., IO_3^- , IO_2^-) may explain this additional buffering component. There is no clear way of establishing an equilibrium concentration of these anions in our solutions. However, this does not invalidate this well-established fluorescent procedure because matched controls are always performed (i.e., $\pm$ agonist) and increases in K_q would serve to underestimate influx.

Table 4. Basal halide influx in CFTR -expressing ($\text{CFTR}^{+/+}$, $\text{CFTR}^{+/-}$) and nonexpressing ($\text{CFTR}^{-/-}$) mouse pups

Genotype	$J_{\text{I}^- \text{ anion}}$, mM/s		
	Chloride	Bromide	Iodide
Pup $\text{CFTR}^{+/+}$, $\text{CFTR}^{+/-}$	0.35 ± 0.05	0.33 ± 0.07	0.38 ± 0.1
Adult $\text{CFTR}^{+/+}$, $\text{CFTR}^{+/-}$	0.37 ± 0.07	0.32 ± 0.07	0.45 ± 0.09
<i>n</i>	5	3	15
Pup $\text{CFTR}^{-/-}$	0.25 ± 0.08	0.24 ± 0.05	0.26 ± 0.07
Adult $\text{CFTR}^{-/-}$	ND	ND	ND
<i>n</i>	5	3	15

Values are means $\pm$ SD; *n*, number of animals. $J_{\text{I}^- \text{ anion}}$, halide influx rate; ND, not detected. Rates were calculated in SPQ-loaded crypts using Stern-Volmer quench constants (K_q) derived in Fig. 1.

With the calculated K_q value for I^- , we tabulated changes in agonist-evoked I^- influx rate ($\Delta J_{\text{I}^- \text{ agonist}}$) in response to FSK ($10 \mu\text{M}$), CCh ($500 \mu\text{M}$), and NSP4 peptide (500 nM) in all three CFTR genomic backgrounds (Table 5). Time-based histograms of changes in agonist-induced quench rate [$\Delta R_{\text{I}^-} = (\Delta F/\Delta t_{\text{agonist}} - \Delta F/\Delta t_{\text{basal}})$] are also shown (Figs. 5–7).

FSK-stimulated I^- influx into crypt cells was age independent and required CFTR . FSK, as predicted from previous studies (10, 45), induced significant I^- influx in both $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ mouse pup and adult crypts; $J_{\text{I}^- \text{ agonist}}$ increased 3.7- to 4-fold over $J_{\text{I}^- \text{ basal}}$ and were statistically significant ($P < 0.001$, $n = 5$; Table 5). Age-dependent differences in the magnitude of ΔJ_{I^-} were absent ($P > 0.05$, $n = 5$; Table 5). Time-dependent analysis revealed that the peak FSK-induced quenching rate ($R_{\text{I}^- \text{ agonist}}$) occurred 8–10 s after bath I^- exchange (Fig. 5A).

In contrast, when crypts isolated from $\text{CFTR}^{-/-}$ mouse pup littermates were exposed to FSK, much lower $J_{\text{I}^- \text{ agonist}}$ and ΔJ_{I^-} values were obtained. In this instance, $J_{\text{I}^- \text{ agonist}}$ failed to be significantly different from basal J_{I^-} values ($P > 0.05$) and both ΔJ_{I^-} and ΔR_{I^-} values were significantly lower than corresponding values obtained from $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ mouse pup crypts (ΔJ_{I^-} $\text{CFTR}^{-/-} = 0.03 \pm 0.02 \text{ M}^{-3}/\text{s}$ vs. ΔJ_{I^-} $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-} = 1.4 \pm 0.46 \text{ M}^{-3}/\text{s}$, $P < 0.001$, $n = 5$; Table 5 and Fig. 5B). The absence of CFTR was correlated with $>95\%$ inhibition of FSK-stimulated I^- influx into isolated mouse pup crypts. In all genotypes, FSK stimulated changes in influx in $\sim 90\%$ of crypts tested.

CCh-stimulated I^- influx into crypt cells was age independent and required CFTR expression. CCh ($100 \mu\text{M}$) $J_{\text{I}^- \text{ agonist}}$ values were twofold greater than $J_{\text{I}^- \text{ basal}}$ values in wild-type and heterozygous CF genotypes and were significantly different ($P < 0.001$, $n = 5$; Table 5). The resulting ΔJ_{I^-} values from both groups displayed no age dependency (ΔJ_{I^-} pup = $0.62 \pm 0.30 \text{ M}^{-3}/\text{s}$ vs. ΔJ_{I^-} adult = $0.69 \pm 0.24 \text{ M}^{-3}/\text{s}$, $n = 5$, $P > 0.05$; Table 5). These changes approximated to 51 and 40% of the values elicited by FSK in pup and adult crypts, respectively (Table 5). CCh-elicited ΔR_{I^-} changes were greatest 8–10 s after bath I^- exchange, with a latency

Table 5. Summary of the effects of forskolin, CCh, and NSP4 on I^- influx rates in $CFTR^{+/+}, +/+$ and $CFTR^{-/-}$ mice

Condition	$CFTR^{+/+}, +/+$					$CFTR^{-/-}$					<i>P</i>
	J_{I^-} basal	J_{I^-} agonist	<i>P</i>	ΔJ_{I^-} net	<i>n</i>	J_{I^-} basal	J_{I^-} agonist	<i>P</i>	ΔJ_{I^-} net	<i>n</i>	
FSK _{pup}	0.48 ± 0.1	1.9 ± 0.56	<0.001	1.4 ± 0.46	5	0.27 ± 0.14	0.3 ± 0.14	NS	0.03 ± 0.02	5	<0.001*
FSK _{adult}	0.43 ± 0.08	1.68 ± 0.5	<0.001	1.25 ± 0.29	5	ND	ND	NS	ND		
<i>P</i>	NS	NS		NS							
CCh _{pup}	0.32 ± 0.08	0.94 ± 0.27	<0.001	0.62 ± 0.3	5	0.19 ± 0.06	0.3 ± 0.1	NS	0.11 ± 0.08	5	<0.001*
CCh _{adult}	0.53 ± 0.14	1.22 ± 0.32	<0.001	0.69 ± 0.24	5	ND	ND	NS	ND		
<i>P</i>	NS	NS		NS							
NSP4 _{pup}	0.4 ± 0.14	1.07 ± 0.32	<0.001	0.67 ± 0.24	5	0.22 ± 0.05	0.66 ± 0.13	<0.001	0.43 ± 0.1	5	NS*
NSP4 _{adult}	0.6 ± 0.13	0.67 ± 0.14	NS	0.06 ± 0.02	5	ND	ND		ND		
<i>P</i>	NS	<0.001		<0.001							
NSP4 _{pup} -EGTA	0.32 ± 0.08	0.3 ± 0.06	NS	-0.02 ± 0.06	4						
NSP4 _{pup} -DIDS	0.29 ± 0.13	0.34 ± 0.14	NS	0.05 ± 0.06	4						

Values are means ± SD expressed as mM/s. FSK, forskolin; J_{I^-} basal, basal I^- influx rate; J_{I^-} agonist, agonist-stimulated I^- influx rate; ΔJ_{I^-} net, net agonist-stimulated I^- influx rate; ND, not determined; NS, not significant ($P > 0.05$). **P*, unpaired Student's *t*-test of ΔJ_{I^-} net $CFTR^{+/+}, +/+$ vs. $CFTR^{-/-}$.

similar to that recorded for FSK (Fig. 6A). CCh was therefore a potent age-independent stimulator of plasma membrane I^- influx in $CFTR$ -containing crypts, causing identical but smaller changes in I^- influx rate compared with FSK.

Like FSK, CCh failed to significantly increase the plasma membrane I^- influx rate in $CFTR^{-/-}$ mouse pup

crypts. J_{I^-} agonist and J_{I^-} basal values failed to be statistically different ($P < 0.05$, Student's *t*-test, $n = 5$; Table 5). The ΔJ_{I^-} recorded in CF mouse pup crypts was significantly smaller than $CFTR^{+/+}$ and $CFTR^{+/+}$ litter-

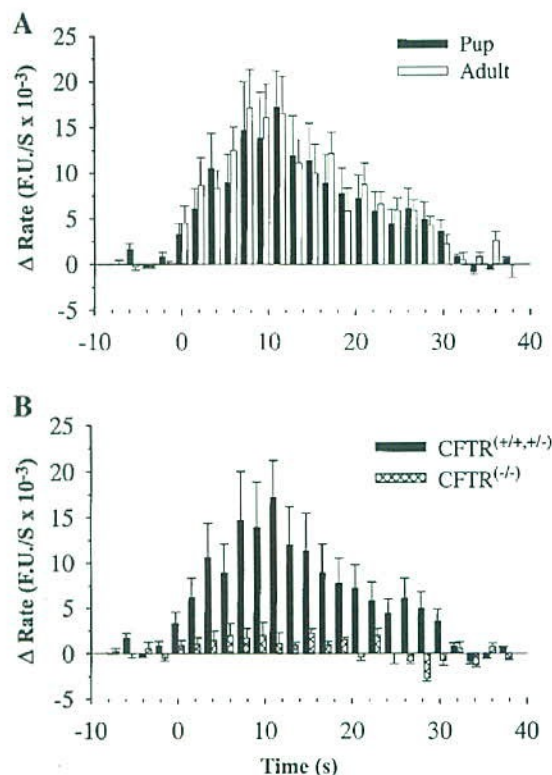


Fig. 5. Change in SPQ fluorescence quench rate in mouse distal colon crypts elicited by cAMP-mobilizing agonist forskolin (FSK, 10 μ M). A: values obtained from adult and pup $CFTR^{+/+}$ and $CFTR^{+/+}$ mouse crypts, showing a lack of any age dependency. B: values obtained from pooled $CFTR^{+/+}$ and $CFTR^{+/+}$ and $CFTR^{-/-}$ pup crypts, demonstrating that effects of FSK on SPQ quench rate were dependent on $CFTR$ expression. Values are means ± SD. FU, fluorescence unit.

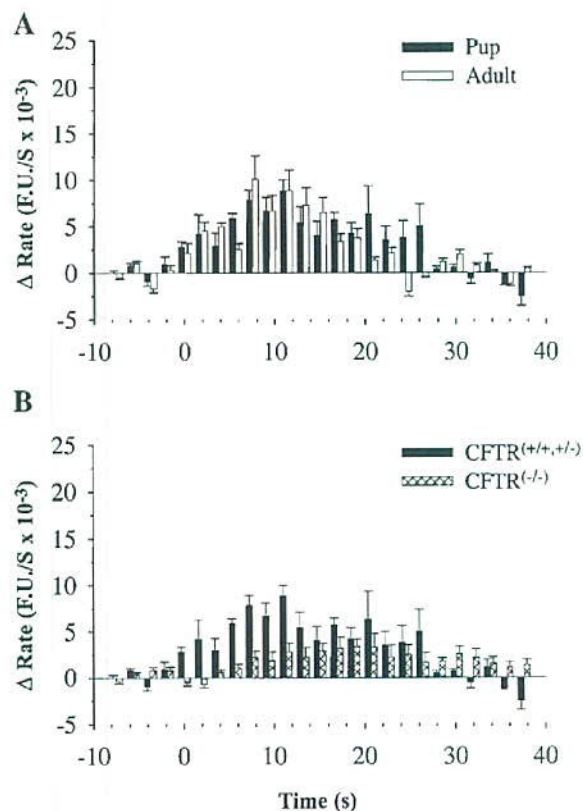


Fig. 6. Change in SPQ fluorescence quench rate in mouse distal colon crypts elicited by Ca^{2+} -mobilizing agonist CCh (100 μ M). A: values obtained from pooled adult and pup $CFTR^{+/+}$ and $CFTR^{+/+}$ mouse crypts, showing a lack of any age dependency. B: values obtained from pooled $CFTR^{+/+}$ and $CFTR^{+/+}$ and $CFTR^{-/-}$ pup crypts, demonstrating that effects of CCh on SPQ quench rate were delayed and significantly smaller in magnitude when $CFTR$ was absent from crypt. Values are means ± SD.

mate values ($\text{CFTR}^{-/-} \Delta J_{\text{I}^- \text{ agonist}} = 0.11 \pm 0.08 \text{ M}^{-3}/\text{s}$ vs. $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-} \Delta J_{\text{I}^-} = 0.62 \pm 0.3 \text{ M}^{-3}/\text{s}$, $P < 0.001$, $n = 5$; Table 5). When ΔR_{I^-} for CCh was plotted by histogram analysis in the $\text{CFTR}^{-/-}$ background, quite different kinetics were observed. Both the onset and subsequent peak in ΔR_{I^-} trailed wild-type and heterozygous values by 4 and 18 s, respectively (Fig. 6B). This delay was not well defined by the single exponential fitted at *time 0* during initial rate analysis (J_{I^-}). Thus time-based histograms of rate change are shown. Peak values of CCh-induced ΔR_{I^-} recorded from $\text{CFTR}^{-/-}$ mouse pup crypts approximated to 60% of values recorded from wild-type and heterozygous genotypes. In all genotypes, CCh stimulated changes in $\sim 65\%$ of crypts tested. We therefore concluded that CCh, like FSK, requires CFTR to exert its major effects on plasma membrane I^- influx. Rate analysis revealed, however, that a slower component of SPQ quench rate was also present when CCh, but not FSK, was added to crypts from $\text{CFTR}^{-/-}$ pups.

NSP4-stimulated I^- influx into crypt cells was age dependent and largely independent of CFTR expression. NSP4 protein (100 nM) was found to elicit changes in plasma membrane I^- influx in crypts isolated from wild-type and heterozygous mouse CFTR genotypes. However, unlike either FSK or CCh, the effects of NSP4 were clearly age dependent (Table 5). In the pup crypt, NSP4 stimulated plasma membrane I^- influx by 2.7-fold, whereas, in the adult crypt, NSP4-stimulated I^- influx was <1.1 -fold. Corresponding P values for $J_{\text{I}^- \text{ agonist}}$ vs. $J_{\text{I}^- \text{ basal}}$ were significantly different for pup ($P < 0.001$) but not adult ($P > 0.05$) crypts. Net changes in NSP4-elicited influx rate were as follows: ΔJ_{I^-} pup = $0.67 \pm 0.42 \text{ M}^{-3}/\text{s}$ vs. ΔJ_{I^-} adult = $0.06 \pm 0.02 \text{ M}^{-3}/\text{s}$, and were significantly different ($P < 0.001$, $n = 5$; Table 5). When the NSP4-induced change in I^- influx rate was displayed in histogram format, peak changes in influx rate had a lag of 10 s after introduction of I^- into the bath, similar to that recorded for both FSK and CCh (Fig. 7A).

Significantly, when NSP4 protein (100 nM) was tested on $\text{CFTR}^{-/-}$ mouse pup crypts, a threefold increase in I^- influx rate was recorded. $J_{\text{I}^- \text{ agonist}}$ was statistically different from $J_{\text{I}^- \text{ basal}}$ ($P < 0.001$) and was not statistically different from $J_{\text{I}^- \text{ agonist}}$ values obtained in wild-type and heterozygous CFTR backgrounds ($P > 0.05$; Table 5). The net change in NSP4-induced I^- influx rate approximated to 64% of $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ littermate values ($\Delta J_{\text{I}^-} \text{ CFTR}^{-/-} = 0.43 \pm 0.1$ compared with $\Delta J_{\text{I}^-} \text{ CFTR}^{+/+}$ and $\text{CFTR}^{+/-} = 0.67 \pm 0.42$). The ΔR_{I^-} values failed to exhibit either slow onset or delayed peak (as observed for CCh) in the $\text{CFTR}^{-/-}$ genotype (see above) but instead peaked 10 s after I^- introduction into the bath, paralleling NSP4, FSK, and CCh effects in CFTR-expressing crypts (Fig. 7B). In all genotypes, NSP4 stimulated changes in I^- influx in $\sim 70\%$ of crypts tested, a response rate twice that of NSP4-induced Ca^{2+} mobilization. Thus NSP4-evoked plasma membrane I^- influx was age dependent, occurring selectively in mouse pup crypts, and was

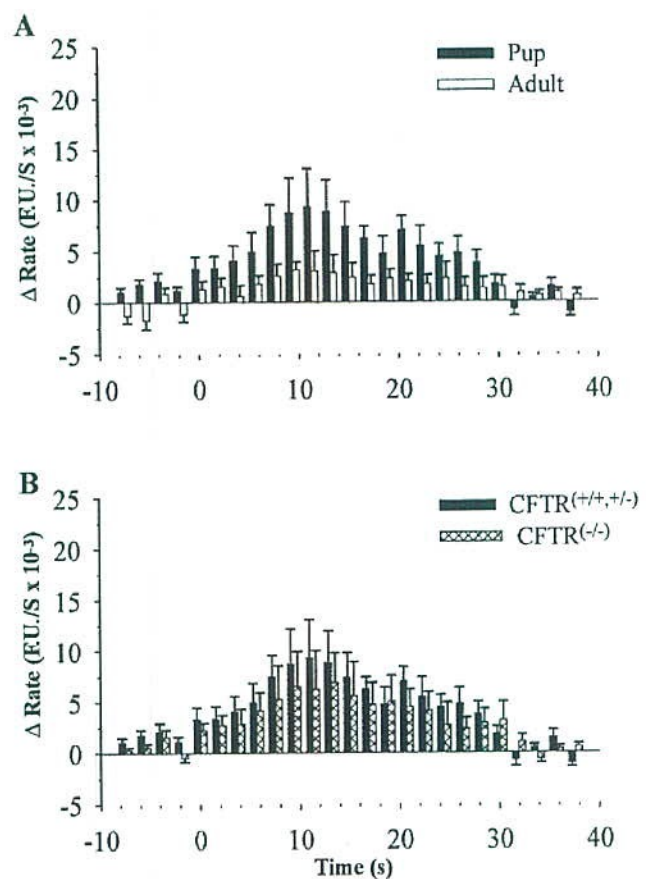


Fig. 7. Change in SPQ fluorescence quench rate in mouse distal colon crypts elicited by NSP4 (100 nM). A: values obtained from pooled adult and pup $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ mouse crypts, demonstrating that NSP4 effects were age dependent. B: values obtained from pooled $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ and $\text{CFTR}^{-/-}$ pup crypts, demonstrating that effects of NSP4 on SPQ quench rate were independent of CFTR expression. Values are means $\pm$ SD.

largely independent of CFTR expression. In crypts from wild-type and heterozygous CFTR pups, the NSP4-induced I^- influx rates were larger but not significantly different (1.56-fold, $n = 5$; Table 5). Thus CFTR expression may additionally be utilized by NSP4 to promote greater overall changes in plasma membrane halide permeability.

NSP4-induced I^- influx into pup crypt cells was dependent on $[\text{Ca}^{2+}]_i$ mobilization. To investigate the relationship between NSP4-induced Ca^{2+} mobilization and plasma membrane I^- influx, $J_{\text{I}^- \text{ basal}}$, $J_{\text{I}^- \text{ agonist}}$, and ΔJ_{I^-} were measured in $\text{CFTR}^{+/+}$ and $\text{CFTR}^{+/-}$ mouse pup crypts loaded and bathed in Ca^{2+} -free HEPES-buffered saline (nominally Ca^{2+} -free plus 1 mM EGTA). Basal I^- influx rate was not appreciably lower in this instance than in crypts bathed with standard (1 mM CaCl_2) extracellular saline ($J_{\text{I}^- \text{ basal}} \text{ Ca}^{2+}\text{-free} = 0.32 \pm 0.08 \text{ M}^{-3}/\text{s}$ vs. $J_{\text{I}^- \text{ basal}} \text{ Ca}^{2+}\text{-containing} = 0.4 \pm 0.14 \text{ M}^{-3}/\text{s}$, values were not significantly different, $P > 0.05$, $n = 4$ and 15, respectively; Table 5). However, in all crypts the absence of bath Ca^{2+} completely inhibited NSP4-mediated I^- influx ($\Delta J_{\text{I}^-} \text{ Ca}^{2+}\text{-free} = -0.02 \pm 0.06 \text{ M}^{-3}/\text{s}$ vs. $\Delta J_{\text{I}^-} \text{ Ca}^{2+}\text{-containing} = 0.67 \pm 0.42$

M^{-3}/s , $P < 0.001$, $n = 4$ and 5 , respectively; Table 5). These findings demonstrated that NSP4-stimulated plasma membrane I^- influx was dependent on $[\text{Ca}^{2+}]_i$ mobilization.

NSP4 protein-induced I^- influx in mouse pup crypt cells was inhibited by DIDS. The effectiveness of NSP4, but not CCh, at eliciting significant I^- influx into SPQ-loaded CFTR $^{-/-}$ mouse pups raised the question as to the nature of this novel conductive pathway. To begin to characterize this pathway, crypts were preexposed to 0.5 mM DIDS and then washed before experimentation. DIDS inhibits a variety of Cl^- channels identified in epithelial cells, including the outward-rectifying Cl^- channel, the swelling-activated Cl^- conductance channel, and the Ca^{2+} -sensitive Cl^- channel, but does not affect cAMP-stimulated CFTR Cl^- channels (reviewed in Ref. 19). DIDS also inhibits a variety of organic osmolyte exchange mechanisms (52) and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (63). Basal I^- influx rate in the presence of DIDS was lower than controls but was not significantly different ($J_{\text{I}^- \text{ basal DIDS}} = 0.29 \pm 0.13 \text{ M}^{-3}/\text{s}$ vs. $J_{\text{I}^- \text{ basal control}} = 0.4 \pm 0.14 \text{ M}^{-3}/\text{s}$, $P > 0.05$, $n = 5$; Table 5). This general halide transport inhibitor completely abolished the NSP4-elicited increase in plasma membrane I^- influx in all crypts ($J_{\text{I}^- \text{ agonist DIDS}} = 0.34 \pm 0.14 \text{ M}^{-3}/\text{s}$ vs. $J_{\text{I}^- \text{ agonist control}} = 0.107 \pm 0.32 \text{ M}^{-3}/\text{s}$, $P < 0.001$, $n = 4$), and the net change in I^- influx rate reflected this difference ($\Delta J_{\text{I}^- \text{ DIDS}} = 0.05 \pm 0.06$ vs. $\Delta J_{\text{I}^- \text{ control}} = 0.67 \pm 0.24$, $P < 0.001$, $n = 4$; Table 5). Thus the ontogenically regulated NSP4-activated plasma membrane I^- influx pathway present in mouse pup crypts was DIDS sensitive.

DISCUSSION

Relationship between the diarrhea effects of infectious rotavirus and NSP4. The induction of diarrhea by NSP4 protein and NSP4₁₁₄₋₁₃₅ peptide compared with active rotavirus indicates that the enterotoxigenic effects of NSP4 may account for the initial diarrheal phase of rotaviral infection (3). During this period, diarrhea occurs without significant ultrastructural damage to the small intestinal mucosa (38, 57). This contrasts with diarrhea seen during the later stages of rotaviral infection in some animal species, in which villus blunting has been suggested to contribute to diarrhea by reducing small intestinal absorptive area (5). This later histopathological feature of rotaviral infection is not seen in all animal models; in the mouse, significant villus blunting is rare (38, 49). Therefore, loss of absorptive area is likely to be auxiliary to the underlying pathophysiological basis of diarrhea in this model. Supporting this hypothesis, we have shown that NSP4 and its active NSP4₁₁₄₋₁₃₅ peptide fail to cause significant histological damage to the mucosa when injected intraluminally or interperitoneally into wild-type mice (unpublished observations and Ref. 3).

Correlation between $[\text{Ca}^{2+}]_i$ mobilization and fluid transport in the gut. Addition of Ca^{2+} -mobilizing agonists such as CCh to intestinal mucosal sheets from either CFTR $^{-/-}$ mice or CF patients fails to evoke a Cl^- secretory current (6, 37, 53). This lack of response

suggests that the cellular effects of Ca^{2+} mobilization on the Cl^- secretory current in the gastrointestinal tract occur secondary to cAMP-dependent activation of CFTR channels and are limited to the upregulation of basolateral membrane ion transporters and K^+ channels (51, 60). Results from NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization in both native small and large intestinal crypts (Fig. 2, A–C, Fig. 3, and Table 2) support the hypothesis that the enterotoxigenic activity of NSP4 in normal mucosa may be partly due to the secondary effects of increased cellular Cl^- uptake at the basolateral plasma membrane and cellular hyperpolarization. These facilitating but not controlling mechanisms for intracellular Cl^- conduction across the luminal membrane do not explain all of our observations. Two important questions relating to the role of NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization, namely the age-dependent effects of NSP4 on Cl^- secretion and diarrhea and the diarrheal effects of NSP4 in CF mice, remained unanswered.

NSP4- Ca^{2+} signaling does not directly regulate age-dependent Cl^- secretion. If changes in $[\text{Ca}^{2+}]_i$ levels alone were solely responsible for Cl^- secretion and diarrhea in mouse pups, then Ca^{2+} mobilization by NSP4 would be expected to be cell differentiation and/or crypt age dependent. Our studies found, however, that NSP4 mobilized $[\text{Ca}^{2+}]_i$ equally well in all crypt regions and was therefore independent of cellular maturity (Fig. 4). These findings contrast with the reported effects of both purinergic and muscarinic agonists on $[\text{Ca}^{2+}]_i$ in intestinal crypts, which show greater responsiveness in the basal region, where Cl^- secretion is believed to occur (29, 47). The more widespread $[\text{Ca}^{2+}]_i$ mobilization elicited by NSP4 would argue that antiabsorptive as well as prosecretory effects may be present in rotaviral-infected intestinal mucosa (see *An antiabsorptive model for NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization*). Furthermore, the effects of NSP4 on $[\text{Ca}^{2+}]_i$ mobilization were neither strain dependent nor related to the age of the animals from which the crypts were isolated (Table 3). These findings therefore indicated that NSP4-mediated Ca^{2+} mobilization cannot directly account for the age dependency to Cl^- secretion and therefore secretory diarrhea reported by us in neonatal mice (3).

NSP4- Ca^{2+} signaling activates an age-dependent anion permeability in normal and CF mice. Both CCh and NSP4 have been shown to utilize PLC-dependent IP_3 signaling to elevate $[\text{Ca}^{2+}]_i$ (13). We therefore examined how NSP4, but not CCh, induces diarrhea in CF mouse pups. The SPQ fluorescence imaging technique has been used previously to measure basal and agonist-stimulated Cl^- permeability changes in both freshly isolated crypts (22) and primary cultures of rabbit (10, 45, 46) and human (45) distal colon. Cl^- transport measurements made with SPQ have demonstrated the absence of CFTR from epithelial cells of CF patients (41, 42, 50), and modeling studies have shown that the SPQ technique provides equivalent information to that obtained from either electrophysiological or isotopic flux studies (59).

Whereas both FSK and CCh elicited age-independent increases in I^- influx that are dependent on the

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presence of CFTR (Table 5, Fig. 5, and Fig. 6, A and B), the effects of NSP4 were age dependent and largely CFTR independent (Table 5). Both CCh and NSP4 protein were more effective at causing plasma membrane permeability changes than causing generalized $[\text{Ca}^{2+}]_i$ mobilization. We believe this reflects both our technical limitations at isolating crypts with endogenous levels of receptor activity (see RESULTS) and the fact that Ca^{2+} -mobilizing agonists can couple efficiently with plasma membrane anion channels through localized $[\text{Ca}^{2+}]_i$ rises at the level of the plasma membrane that often do not provoke a generalized $[\text{Ca}^{2+}]_i$ mobilization (35).

The lack of an age dependency for CFTR-dependent I^- permeability changes corroborated Cl^- transport studies made in weaning and adult rat and pig colon but differed from the age-dependent effects of cGMP or *Escherichia coli* heat-stable enterotoxin (STa) in primary cultures of weaning and adult rabbit colon. In these instances agonist responses were greater in crypts isolated from older mice (10). Differences may, however, reflect the fact that Cl^- was used as a quencher in the latter studies, and rates may represent both conductive and nonconductive components of plasma membrane Cl^- permeability. In addition, STa effects on transmucosal Cl^- secretion and crypt Cl^- permeability are absent in the CF mice (20). Thus NSP4 utilizes a different plasma membrane permeability pathway in distal colon crypts from that utilized by STa or its mammalian counterparts, guanylin or uroguanylin, and other nitroso compounds of nitric oxide-signaling pathways.

The age dependency to NSP4-elicited changes in plasma membrane I^- influx suggests that NSP4-elicited diarrhea in CF mouse pups (Table 1) may be brought about by the activation of a novel cellular permeability pathway. The fact that neither FSK nor CCh promoted similar changes in CF mice, and that NSP4-induced I^- permeation was both Ca^{2+} dependent and blocked by DIDS (Table 5), supported the hypothesis that we are reporting on a different permeability pathway. NSP4-induced I^- influx into CFTR $^{-/-}$ mouse pup crypts was ~60% of that elicited in the wild-type and heterozygous background. This difference may possibly explain the higher, but not significantly different, incidence of diarrhea reported for infectious rotavirus, NSP4 protein, and NSP4₁₁₄₋₁₃₅ peptide in CFTR $^{+/+}$ and CFTR $^{+/-}$ mice. NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization would be expected to facilitate both CFTR-dependent and CFTR-independent anion permeation by upregulating Ca^{2+} -dependent secondary active transport mechanisms at the basolateral membrane.

Our findings would also provide a functional explanation for the recent observations of Angel and colleagues (2), who demonstrated that CF mice and their non-CF siblings exhibit diarrhea after rotaviral infection. The age- and Ca^{2+} -dependent changes in intestinal epithelial cell plasma membrane anion permeability evoked by NSP4, shown in this study to be CFTR independent, may contribute to (or constitute) the acute phase of the rotaviral diarrheal response. This cellular phenomena

would precede immune/morphological changes occurring within the mucosa after viral infection.

Candidate anion conductances for NSP4-induced age-dependent permeability changes in the mouse pup crypt. Although the plasma membrane halide permeability changes promoted by NSP4 were clearly Ca^{2+} dependent and could be inhibited pharmacologically, our investigations have yet to confirm the biophysical nature of the conductance. Furthermore, the SPQ technique does not allow us to determine the plasma membrane location of the NSP4-elicited I^- influx. Future studies utilizing alternate methods should address these questions. Even so, at this point, it is of interest to consider candidate conductances that may participate in this phenomena.

Direct activation of apical plasma membrane Ca^{2+} -sensitive Cl^- channels by classical PLC-dependent Ca^{2+} -mobilizing secretagogues, although clearly demonstrated in gastrointestinal cell lines and epithelial cells from other organs, e.g., the lungs (19), has not been shown in native intestinal epithelia. However, both enteroendocrine and macrophage/lymphocyte populations of the gut are known to secrete cryptdin proteins, some of which are believed to act themselves as anion conductances (30), whereas others, notably the defensins, are proposed to act at nanomolar concentrations to stimulate Ca^{2+} -dependent Cl^- secretion (4, 31). We therefore propose that NSP4 either 1) itself creates a conductance in the apical plasma membrane, 2) activates a dormant Ca^{2+} -activated anion channel within this membrane with biophysical properties similar to those recorded in vitro (reviewed in Ref. 19) through its stimulatory effects on PLC-induced $[\text{Ca}^{2+}]_i$ mobilization (13), or 3) acts as a regulatory protein at the level of Ca^{2+} - or anion-channel signaling to elicit Ca^{2+} -dependent permeability changes across the cellular plasma membrane. CCh failed to promote similar changes in the initial rate of I^- influx into CF mouse pup crypts as NSP4 but elicited a response with a delayed onset, consisting of ~20% of wild-type and heterozygous CFTR values (Table 5 and Fig. 6B). Because supramaximal concentrations of CCh were used, these differences do not reflect dose dependency and argue against the presence of an ontogenically regulated conductance that can be stimulated by all Ca^{2+} -mobilizing agonists. The delayed onset changes in I^- permeability promoted by CCh (Fig. 6B) are unlikely to be localized to the apical membrane because it is clear that CCh fails to elicit a Cl^- secretory response in CF mucosal sheets (6, 37, 53).

A possibility exists that NSP4 may activate a native cell counterpart of osmotically regulated Cl^- channels. These conductances, although not directly activated by Ca^{2+} , are indirectly Ca^{2+} sensitive and have been shown to participate in regulatory volume decreases (RVD) in intestinal epithelia (12). These channels, believed to be localized on the basolateral plasma, participate in the maintenance of fluid and osmolyte absorption. They are functionally expressed in the surface and neck regions of the crypt as well as the small intestinal villi, areas known to exhibit RVD (11)

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and have been shown to be impaired in $\text{CFTR}^{-/-}$ mice (55). Our SPQ fluorescent measurements were made, however, in lower crypt regions, which do not undergo RVD. Thus they are poor candidates for the NSP4 response.

Volume changes in response to Ca^{2+} -mediated secretagogues have been reported in CF mouse small intestinal crypts (56), suggesting that additional conductive pathways exist. The slow onset response observed for I^- influx after CCh challenge in $\text{CFTR}^{-/-}$ mouse crypts may reflect this alternate pathway (Fig. 6B). However, the timing of the effects of NSP4 in the $\text{CFTR}^{-/-}$ mouse crypt was very different (Fig. 7B), mimicking the profile observed for activation of apical membrane CFTR in wild-type crypts by all agonists (Figs. 5A, 6A, and 7A). We interpret these results to suggest that the effects of NSP4 on any basolateral membrane permeability, if present, are likely to be secondary phenomena and unrelated to the primary effects of NSP4 on the apical plasma membrane I^- influx.

A prosecretory model for NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization. A characteristic property of rotavirus infection is that it causes age-dependent gastroenteritis in neonatal mammals (25). In the neonatal mouse, increases in small and large intestinal luminal Na^+ , Cl^- , and water content have been correlated with diarrheal activity during rotaviral infection (49). Previously, we have reported (3) that NSP4 protein addition to mouse pup small intestinal mucosal sheets mimics the transepithelial Cl^- secretory effects of CCh, an endogenous Ca^{2+} -mobilizing agonist, but that this secretory response was lost in adult tissues. Further strengthening our hypothesis that enterocytes express an ontogenically regulated, Ca^{2+} -activated ionic conductance that underlies the diarrheal response, we now report that NSP4 induces Ca^{2+} -dependent, DIDS-inhibitable I^- influx in $\text{CFTR}^{-/-}$ mouse pup crypts. This phenomenon did not occur in adult crypts. The primary effect of NSP4 may therefore be to activate an apical plasma membrane Ca^{2+} -dependent Cl^- conductance in mouse pup enterocytes with biophysical properties similar to those described in vitro (19). The secondary effect of NSP4, like that of CCh, may be to facilitate CFTR-mediated fluid transport by upregulating Ca^{2+} -sensitive basolateral Cl^- uptake and cellular hyperpolarization. Combined, both may account for the Ca^{2+} -dependent fluid secretory response of normal and CF mucosa and may delineate a secretory component to rotaviral diarrhea.

An antiabsorptive model for NSP4-induced $[\text{Ca}^{2+}]_i$ mobilization. NSP4 mobilized Ca^{2+} in all regions of the crypt from both the small intestine and colon. We have also found that NSP4 is equally effective at mobilizing Ca^{2+} in dispersed villus epithelial cells from the small intestine (data not shown). Basal electroneutral NaCl absorption into the villus has been shown to be inhibited by Ca^{2+} -dependent processes, including PLC- γ , calmodulin, and protein kinase C activation (14, 17, 26). Thus another predicted secondary effect of NSP4-induced Ca^{2+} mobilization is downregulation of this absorptive process. Released NSP4 protein, if it encounters luminal receptors on neighboring cells (3), would

be expected to promote similar antiabsorptive changes. NSP4-induced Ca^{2+} mobilization would not, however, be expected to affect absorptive Na^+ -coupled glucose-solute transport (62), which is the cellular basis of oral rehydration therapy given during bacterial and viral gastroenteritis (40).

We have shown that the nonstructural protein of rotavirus NSP4 and its peptide both elevate intestinal cell $[\text{Ca}^{2+}]_i$ levels and induce diarrhea in neonatal mice with either wild-type or CFTR -deficient genotypes. Ca^{2+} mobilization may effect a diarrheal response in these cells through a variety of cellular mechanisms, including the activation of an ontogenically regulated, Ca^{2+} -sensitive, Cl^- -conductive pathway in small intestinal crypts and Ca^{2+} -inhibitable Na^+ -absorptive processes in small intestinal villi and crypts. Fluid loss by any of these mechanisms would occur independently of CFTR function. The diarrheal properties of NSP4 indicate that molecules based on the active domain of NSP4 may prove useful for the treatment of neonatal meconium ileus syndrome or the less severe clinical condition of constipation, which is prevalent in CF patients (44). The low dose (nanomolar) effectiveness of the NSP4 peptide and protein (3, 13), coupled with the fact that attenuated NSP4 proteins with differing diarrheal activities have been recently identified by us (64), suggests that further molecular characterization of the active domain of NSP4 may help design chemical structures with therapeutic value.

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Inhibition of Cellular Protein Secretion by Norwalk Virus Nonstructural Protein p22 Requires a Mimic of an Endoplasmic Reticulum Export Signal

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Abstract

Protein trafficking between the endoplasmic reticulum (ER) and Golgi apparatus is central to cellular homeostasis. ER export signals are utilized by a subset of proteins to rapidly exit the ER by direct uptake into COPII vesicles for transport to the Golgi. Norwalk virus nonstructural protein p22 contains a YXΦESDG motif that mimics a di-acidic ER export signal in both sequence and function. However, unlike normal ER export signals, the ER export signal mimic of p22 is necessary for apparent inhibition of normal COPII vesicle trafficking, which leads to Golgi disassembly and antagonism of Golgi-dependent cellular protein secretion. This is the first reported function for p22. Disassembly of the Golgi apparatus was also observed in cells replicating Norwalk virus, which may contribute to pathogenesis by interfering with cellular processes that are dependent on an intact secretory pathway. These results indicate that the ER export signal mimic is critical to the antagonistic function of p22, shown herein to be a novel antagonist of ER/Golgi trafficking. This unique and well-conserved human norovirus motif is therefore an appealing target for antiviral drug development.

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Introduction

Maintenance of cellular homeostasis is directly dependent on the proper functioning of the Golgi apparatus, which is central to lipid trafficking and protein secretion. Protein trafficking from the endoplasmic reticulum (ER) to the Golgi is mediated by vesicles coated in COPII protein complexes, whereas the retrograde Golgi-to-ER pathway is mediated by COPI-coated vesicles [1]. Upon export from the ER at ER exit sites (ERES), cellular proteins accumulate and traffic into budding COPII vesicles, which are minimally composed of the GTPase Sar1 and heteromeric complexes of Sec13/31 and Sec23/24 [2,3]. COPII vesicles then traffic along microtubules through the ER/Golgi intermediate compartment to the *cis* Golgi, where vesicles lose their COPII coat, fuse with the Golgi, and progress to the *trans* Golgi [4–6]. A subset of cellular and viral proteins that rapidly exit the ER employ either di-hydrophobic [7], di-basic [8] or di-acidic [9,10] ER export signals that mediate their specific uptake into COPII vesicles by direct interaction with either Sec24 or Sar1 at ERES. Export of proteins from the ER and subsequent trafficking of COPII vesicles to the Golgi is mediated by a number of cellular factors, and proteins of both cellular and microbial origin are known to antagonize this pathway.

Perhaps the most well-known ER/Golgi trafficking antagonist, the fungal metabolite brefeldin A (BFA) targets the GTPase ADP-ribosylation factor 1 (Arf1) responsible for COPI vesicle budding at the Golgi by stabilizing an Arf/Sec7 intermediate during nucleotide exchange [11]. This prevents nucleotide dissociation and ultimately deactivates Arf1 to induce a global inhibition of cellular protein secretion. The 3A proteins encoded by the picornaviruses coxsackievirus B3 (CVB3) and poliovirus (PV) also target Arf1. 3A inhibits GBF1, a guanine exchange factor necessary for Arf1 activation [12,13], resulting in Golgi disruption and inhibition of protein secretion. Consequently, surface expression of MHC Class I decreases and the normal cytokine release that aids in clearance of infected cells is inhibited [14–16]. This results in a prolonged period of viral replication before the infected cell can be cleared by the immune system [12,15].

Human noroviruses are the causative agent of approximately 23 million annual cases of gastroenteritis in the U.S. and are classified as Category B biodefense pathogens [17,18]. Noroviruses are composed of five genogroups within the family *Caliciviridae*, and viruses in genogroups I (GI) and II (GII) are the most frequently detected in humans [19–21]. Noroviruses code for six nonstructural and two structural proteins [22]; however, one of these

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proteins, the nonstructural protein p22, has no identified function in any calicivirus, although an early study on the immune response following infection with Norwalk virus (NV), the prototype human norovirus and calicivirus, demonstrated an immune response directed against p22 in convalescent sera [23]. The study of p22 and other human norovirus proteins is complicated by the lack of both an efficient tissue culture system to grow noroviruses and a reverse genetics system to directly examine protein function during viral infection.

Replication of two cultivatable animal caliciviruses, feline calicivirus (FCV) and murine norovirus (MNV), induces cellular membrane rearrangements as well as alterations in Golgi architecture [24,25], suggesting that Golgi disassembly may be a common consequence of infection. In support of this, FCV p30, a homologue of NV p22, is membrane associated and independently induces ultrastructural changes in several secretory pathway organelles [26], thus proposing ER-derived membranes as a source of membranes to anchor viral genome replication. Similarly, Fernandez-Vega and colleagues demonstrated that the NV nonstructural protein p48 induces Golgi disassembly [27]; however, the possibility of additional viral proteins contributing to alterations in Golgi phenotype and antagonism of protein secretion, as is the case for several picornaviruses [28,29], has not been examined.

In the current study, we asked if the Golgi rearrangements observed during animal calicivirus and picornavirus replication also occur during human norovirus replication, and if p22 has a role in this process. We discovered that p22 has a highly conserved motif that mimics a traditional di-acidic ER export signal and is required for inhibition of ER/Golgi trafficking. This represents a novel approach to antagonize ER/Golgi trafficking, as no other

cellular or microbial protein has been described to use a motif similar to an ER export signal to gain access to and antagonize the secretory pathway.

Results

The Golgi apparatus is disassembled during NV replication

Due to Golgi rearrangements observed during FCV [24] and MNV [25] replication, we first determined if the Golgi is morphologically changed during human NV replication. Golgi integrity was examined in Huh7 cells 24 hours post-transfection (hpt) of NV RNA that results in a single cycle of viral replication [30]. The Golgi was examined in cells that expressed the viral capsid protein VP1, which is made late in replication and serves as a marker for cells replicating NV. As evidenced by the elongated, peri-nuclear re-localization of Golgi marker proteins (asterisks, Figure 1A and B), which are characteristic of the disassembled Golgi observed during mitosis [31,32], the Golgi was disassembled in 53 of 56 (95%) and 42 of 46 (91%) VP1 positive cells based on immunostaining of the *cis* and *trans* Golgi marker proteins GM130 and golgin-97, respectively. This was in contrast to the phenotypically normal, well-compact and condensed Golgi observed in almost all (97%) of non-transfected cells. These results indicate that, like other caliciviruses, NV replication induces disassembly of the Golgi apparatus.

p22 mediates Golgi disassembly and inhibits protein secretion

The NV nonstructural proteins are produced from a self-cleaving polyprotein and are arranged in an order similar to that

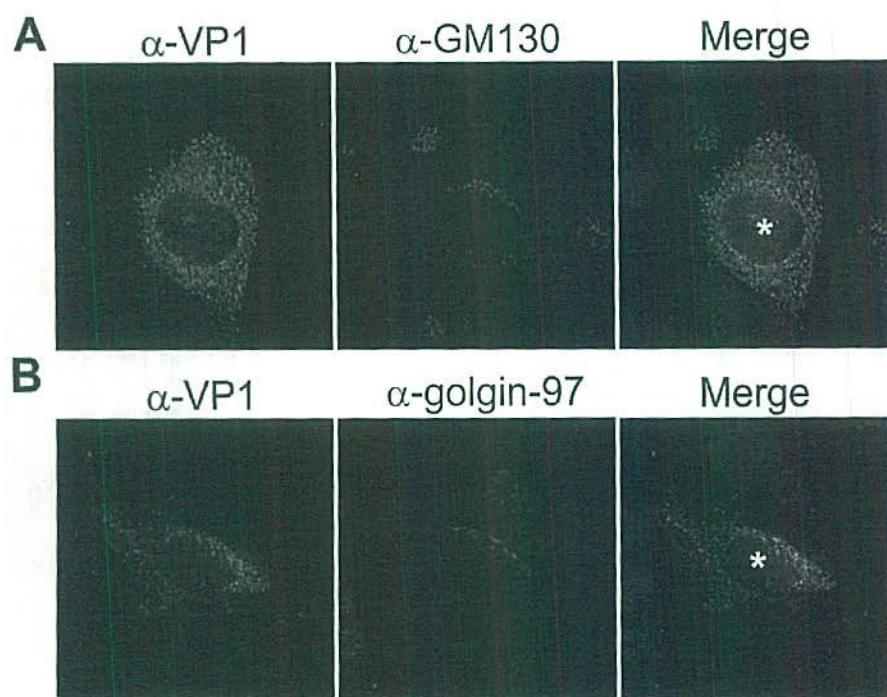


Figure 1. NV replication induces Golgi fragmentation. Viral RNA, purified from the stool of a human volunteer infected with Norwalk virus, was transfected into Huh7 cells grown on coverslips. At 24 hours post-transfection (hpt), cells were fixed and stained for the viral protein VP1 (Alexa 488; green fluorescence) and either the *cis* (A) or *trans* (B) Golgi with antibody against GM130 or golgin-97 (Alexa 594; red fluorescence), respectively. Nuclei were stained with DAPI (blue fluorescence) and imaged by deconvolution microscopy. * indicate cells with disassembled Golgi.
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of the picornavirus polyprotein [33]. p22 is located within this polyprotein in the same location as the picornavirus 3A protein; however, these proteins share no amino acid identity. Although 3A and p22 also differ considerably in molecular weight (10 kDa vs. 22 kDa, respectively) and predicted secondary structure [7% β -sheet and 62% α -helix vs. 13% and 45%, respectively, as predicted by PSIPRED (data not shown)], we hypothesized that p22 contributes to changes in Golgi morphology during Norwalk virus replication, as is the case for the 3A protein, which induces Golgi disruption in several picornaviruses [28,34]. To explore this possibility, the subcellular localization of p22 was characterized by expressing p22 with an N terminal GFP tag in 293T cells by transient transfection, and we used an N terminally GFP-tagged poliovirus 3A protein as a positive control. At 6 hpt, p22 localized to the *cis* Golgi (Figure 2A); however, by 24 hpt p22 showed only minimal *cis* Golgi localization and instead was localized immediately adjacent to a phenotypically disassembled Golgi. Similar results were observed when analyzing the *trans* Golgi (Figure S1).

To characterize the Golgi phenotype in cells expressing 3A and p22, we categorized the morphology of the Golgi in cells as either intact (well-condensed and compact Golgi adjacent to the nucleus),

fragmented (non-compact and peri-nuclear, but easily detectable Golgi; indicated by carets) or dispersed (extremely diffuse and phenotypically unapparent Golgi; indicated by asterisks), the latter two both constituting disassembled Golgi. In cells expressing GFP alone, Golgi fragmentation and dispersion were only observed in a small subset of cells (Figure 2A and B). In contrast, transient expression of the poliovirus 3A protein (PV 3A) induced significant fragmentation and dispersion of the Golgi in 18% ($p = 0.02$) and 62% ($p = 0.003$) of cells, respectively, compared to the Golgi in GFP-transfected cells. Similarly, expression of NV p22 led to a significant increase in the presence of fragmented, though not dispersed, Golgi present in 43% ($p = 0.006$) and 11% ($p = 0.09$) of cells, respectively. The significant differences between 3A and p22 in Golgi fragmentation ($p < 0.01$) and dispersion ($p < 0.003$) suggested that the ultimate effect of p22 on Golgi architecture was markedly different from that of 3A, possibly reflecting a different mechanism to induce Golgi disassembly.

We next sought to determine the biological relevance of the observed Golgi structural alterations on cellular protein secretion. To accomplish this, a secreted alkaline phosphatase (SEAP) reporter assay was utilized in which GFP-tagged p22 and SEAP

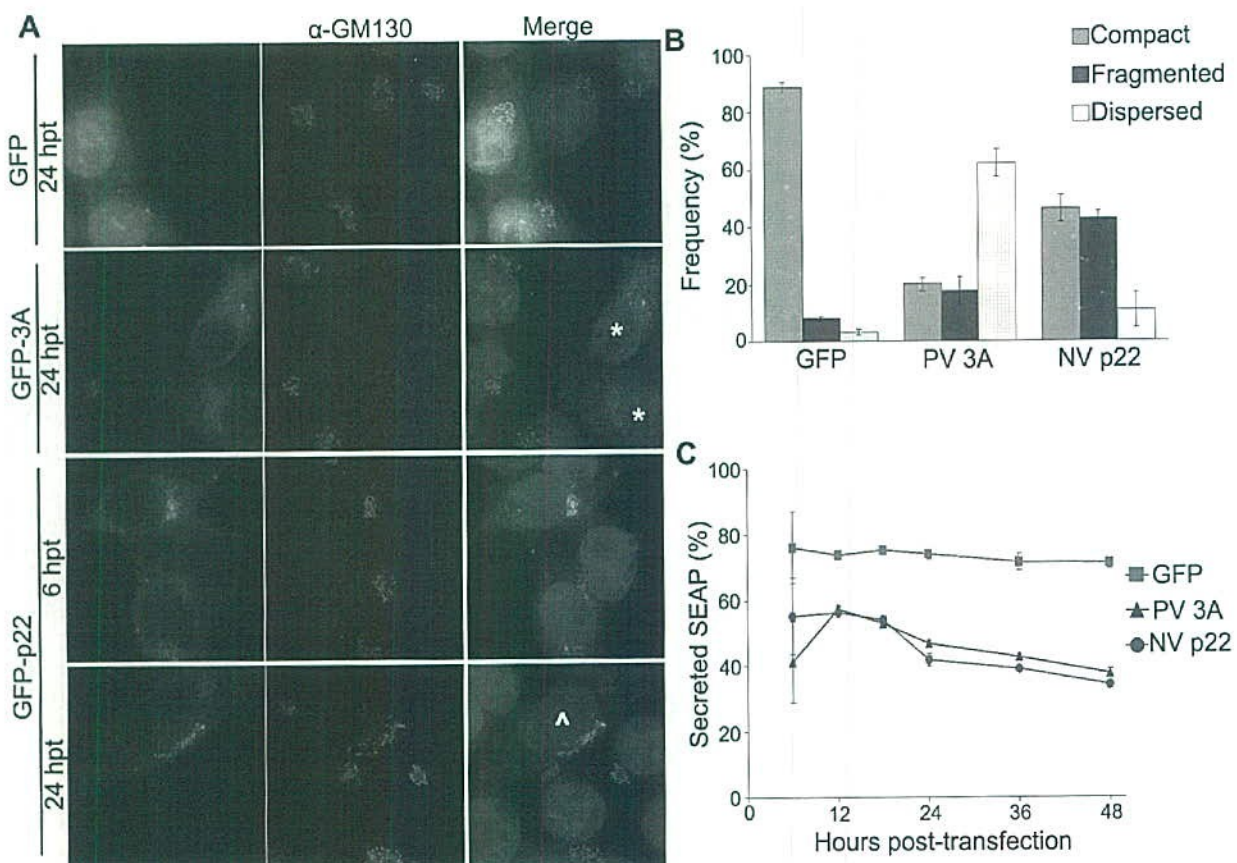


Figure 2. NV p22 induces Golgi disassembly and inhibits protein secretion. (A) Cells expressing GFP, GFP-tagged poliovirus (PV) 3A protein, or GFP-tagged Norwalk virus (NV) p22 were stained for the *cis* Golgi marker protein GM130 (Alexa 594-conjugated secondary antibody, red fluorescence) at the indicated times post-transfection. Nuclei were stained with DAPI (blue fluorescence), and cells were imaged by deconvolution microscopy. * indicates cells with dispersed Golgi; ^ indicates cells with fragmented Golgi. (B) Quantitation of Golgi status in cells expressing the indicated proteins at 24 hours post-transfection ($n = 3$; minimum of 50 cells per experiment; $\pm$ SD). Differences between observed phenotypes are detailed in the text. Results are representative of four independent experiments. (C) Cells were transfected with the plasmid pCMV-UTR-SEAP expressing GFP, GFP-tagged PV 3A, or GFP-tagged NV p22. Secreted SEAP was quantified (see Materials and Methods section) as a representative indicator of cellular protein secretion at the indicated time points, and was defined by the equation: Secreted SEAP = $(SEAP_{extracellular} / (SEAP_{extracellular} + SEAP_{intracellular})) \times 100$. Data are representative of three independent experiments ($n = 3$ for each time point; $\pm$ SD). doi:10.1371/journal.pone.0013130.g002

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were co-expressed from a di-cistronic vector, pCMV-UTR-SEAP [28]. This vector encodes the gene of interest under a CMV promoter and SEAP, a reporter protein that is rapidly secreted from cells and is a quantitative surrogate of protein secretion [35], which is translated via an internal ribosomal sequence. All proteins expressed using this vector system had N terminal GFP tags, which has been reported to not affect the ability of PV 3A to inhibit protein secretion [12]. At various times post-transfection, media and cell pellets were assayed for extra- and intra-cellular enzymatic SEAP activity, respectively; total SEAP levels did not significantly differ between all proteins expressed at any time point tested. For all constructs, enzymatic SEAP activity was first detectable over background in both fractions at 6 hpt. Expression of GFP alone led to ~75% secreted SEAP throughout the assay (Figure 2C), whereas expression of PV 3A led to a significant reduction of SEAP secretion ($p < 0.001$) with maximal reduction to 38%, or 53% of GFP alone levels, at the final time point, which is similar to previous results [13,28,36]. With similar kinetics to 3A, NV p22 also ultimately inhibited SEAP secretion to 34%, or 48% of GFP alone levels. From this, we concluded that, despite their differing specific effects on Golgi phenotype, NV p22 is able to inhibit SEAP secretion, and therefore cellular protein secretion, to levels similar to PV 3A.

To gain a better understanding of potential ultrastructural alterations induced by p22, cells expressing GFP or GFP-p22 were

flow sorted for GFP expression at 24 hpt. After 24 hours of recovery following flow sorting, and therefore 48 hpt, cells were fixed and thin sections were visualized by electron microscopy (EM). After flow sorting, cells expressing GFP alone had intact and peri-nuclearly localized Golgi with cisternal stacks clearly visible in 31 of 59 (52%) cells examined (Figure 3A, arrows). In contrast, cells expressing GFP-p22 had detectable Golgi stacks in just 4 of 57 (7%) cells examined. Instead, GFP-p22 cells exhibited an abundance of large vacuoles, loose single membranes (Figure 3B, asterisk), and double-membrane structures (Figure 3B, arrowheads). Many of these structures had what appeared to be cargo inside them, but the nature of this cargo was unclear as these structures were much larger than would be expected for those containing normal secretory pathway cargo. These results confirmed the immunofluorescence observations of a disassembled and phenotypically abnormal Golgi, demonstrating that expression of p22 led to rearrangements and alterations to various components of the secretory pathway, as would be expected during antagonism of this pathway.

Amino acids 50–148 mediate Golgi localization of p22

We next determined which regions of p22 are responsible for Golgi localization and/or fragmentation. N and C terminal deletion mutants of p22 with N-terminal GFP tags were generated (Figure 4A) with respect to predicted α -helices and β -sheets. GFP-

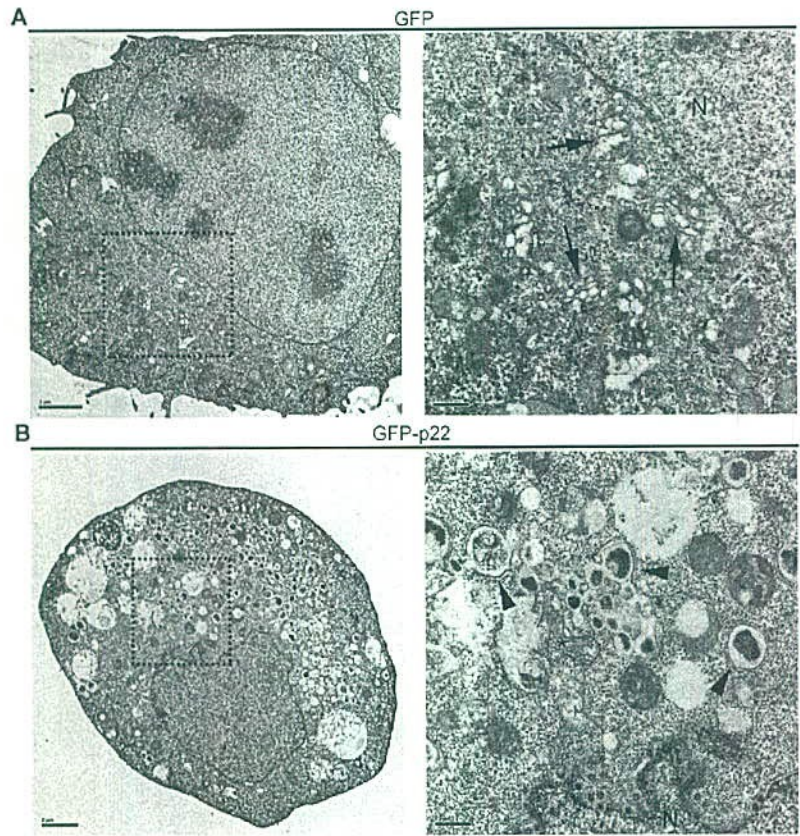


Figure 3. Expression of NV p22 induces alterations in secretory pathway ultrastructure. At 24 hours post-transfection, cells expressing GFP (A) or GFP-p22 (B) were harvested and flow sorted for expression of GFP. Twenty-four hours after re-plating, cells were fixed and prepared for visualization by electron microscopy. The boxed regions represent the area magnified to the right. Left scale bars represent 2 μ m, right scale bars represent 0.5 μ m. N = nucleus; black arrows indicate intact Golgi cisternae; black arrowheads indicate double-membrane vesicles; the asterisk indicates free membranes.
doi:10.1371/journal.pone.0013130.g003

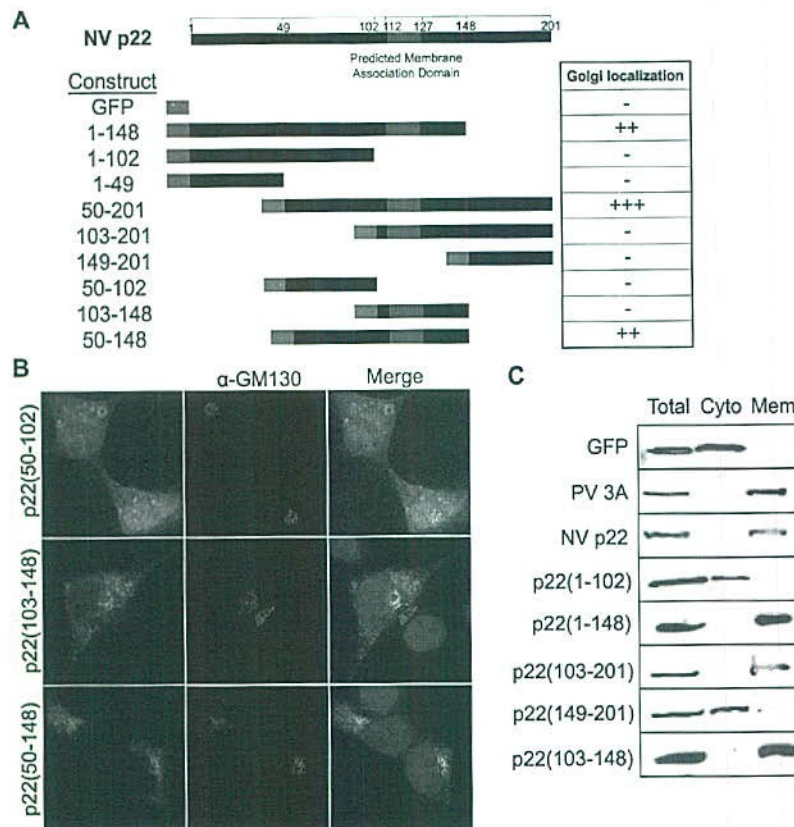


Figure 4. Amino acids 50–148 are sufficient to mediate Golgi localization of NV p22. (A) GFP tagged N and C terminal deletion mutants of p22 were generated and, following expression in cells for 24 hours, were scored for their ability to localize to the *cis* Golgi. Amino acid numbering corresponds to NV sequence (NC_001959). (B) Cells expressing GFP-tagged p22(50–102), p22(103–148), and p22(50–148) were immuno-stained with antibody against the *cis* Golgi marker protein GM130 (Alexa 594-conjugated secondary antibody, red fluorescence), stained with DAPI (blue fluorescence), and imaged by deconvolution microscopy. (C) Cells expressing GFP, GFP-tagged PV 3A, GFP-tagged NV p22, or the indicated deletion mutants of p22 were harvested at 24 hpt. Cytosolic and membranous fractions of cells were collected and proteins were detected by western blot with monoclonal antibody against GFP. doi:10.1371/journal.pone.0013130.g004

tagged deletion mutants encoding amino acids 50–148 localized to the Golgi (summarized in Figure 4A), whereas constructs that lacked either amino acids 50–102 or 103–148 failed to specifically localize to the Golgi; none of the deletion mutants tested were able to induce Golgi disassembly equivalent to wildtype p22. Expression of amino acids 50–102 localized non-specifically throughout cells (Figure 4B), in contrast to amino acids 103–148, which predominantly exhibited a reticular pattern throughout the cytoplasm suggestive of general intracellular membrane localization. When these regions were expressed together as p22(50–148), the protein was predominantly Golgi localized and the Golgi was phenotypically intact.

We next examined the factors between amino acids 50–148 that mediate the subcellular localization of p22. Computational analyses of full-length p22 with PHDhtm (available online at www.predictprotein.org [37]) predicted an amphipathic α -helical trans-membrane (TM) domain between amino acids 112 and 127. A membrane association domain (MAD) within p22 would provide further similarity to the picornavirus 3A protein, which encodes an amphipathic α -helix that acts as both a TM domain and a MAD at the C terminus of the protein [38]. The localization of p22(103–148) and p22(50–148) compared to p22(50–102) (Figure 4B) supported the hypothesis that p22 localizes to the Golgi in part due to membrane association contributed by this domain.

To determine if amino acids 103–148 mediate membrane association of p22, we expressed GFP alone, GFP tagged 3A or p22, or various deletion mutants of p22 in 293T cells and isolated cytosolic and membranous fractions. As expected, GFP was present solely in the cytosolic fraction and PV 3A solely in the membranous fraction of cells (Figure 4C). p22 was also present in the membranous fraction, confirming that it is a membrane-associated protein. Only the p22 deletion mutants that contained amino acids 103–148 were present in the membranous fraction of cells, including a construct encoding amino acids 103–148 alone; all constructs that did not contain amino acids 103–148 were present in the cytosolic fraction. This indicated that residues 103–148 are responsible for membrane association of p22, likely due to an amphipathic α -helix between amino acids 112–127.

p22 contains an ER export signal mimic that is highly conserved in human noroviruses

Because amino acids 103–148 alone resulted in membrane, but not Golgi, localization, we next examined the sequence between amino acids 50–102 for additional factors that might facilitate Golgi localization of p22. A multiple sequence alignment of p22 and its homologues from the 72 available full-length human norovirus sequences from viruses classified in different genogroups (GI.1 to GI.12) found 13% amino acid identity between all

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proteins (summarized in Figure 5A). This defined p22 as the most variable protein in human noroviruses [39]. Despite this, two regions within amino acids 50–148 showed clear sequence conservation. The first region was the MAD (Figure 5A, blue box), indicating that this domain is well-conserved amongst homologues of p22 and likely serves a similar function between genogroups. The second was a YXΦESDG motif (Figure 5A, red box), where X is any amino acid and Φ is a bulky, hydrophobic residue (e.g. M, I or L), which was fully conserved in 65 of the 72 (90%) sequences available for examination (summarized in Figure 5A). Unexpectedly, conservation of this motif was limited to p22 homologues of human noroviruses. It was not present in either the MNV or FCV homologues of p22, both of which exhibit very low amino acid identity with NV p22, and was similarly absent in the 3A protein of poliovirus and coxsackievirus B3. Further examination revealed that the YXΦESDG motif resembled the criteria for a di-acidic ER export signal, which typically contains a YXXΦ motif immediately proximal to two acidic residues separated by a single amino acid [e.g. YXXI(E/D)X(E/

D)], all of which are located on the cytosolic side of a TM domain (Figure 5B) [9,10]. Such signals increase the rate of protein export from the ER into COPII vesicles and onward to the Golgi [9,10,40].

To explore if this conserved norovirus motif could play a role in the previously demonstrated inhibition of cellular protein secretion by p22 (Figure 2C), individual alanine mutations were made within each of the conserved residues within the putative ER export signal of p22 and tested as before using the SEAP system. Mutations within the S, D and G residues had no effect on the ability of p22 to inhibit cellular protein secretion (Figure 5C); however, mutation of both the Y and E residues led to intermediate levels of protein secretion. When these two residues were combined into a single AXΦASDG construct, SEAP secretion at 36 hpt was not statistically different from that of GFP alone ($p = 0.08$). Total SEAP expressed by all p22 mutants did not significantly differ from that of wildtype p22 and similar levels of all p22 proteins were expressed, as confirmed by western blot analysis of intracellular fractions at 36 hpt (Figure S2),

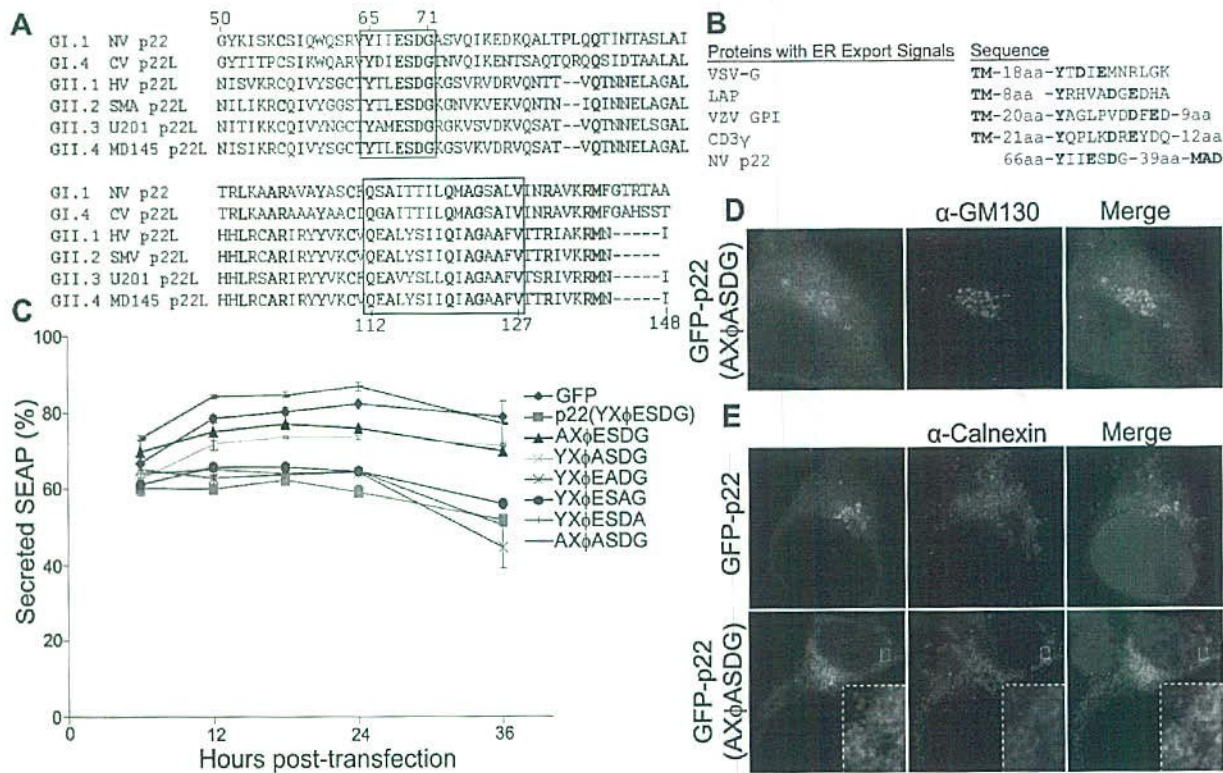


Figure 5. A conserved noroviral ER export signal is necessary for p22 to inhibit protein secretion. (A) Amino acids 50–148 from NV p22 were aligned with homologues (“p22-like (p22L) proteins”) from representative genogroup 1 (GI) and genogroup 2 (GII) human noroviruses of various genotypes. The figure illustrates six of 72 sequences analyzed. Conserved residues are shown in bold; the blue box indicates conservation of the membrane association domain (MAD); the red box indicates conservation of the YXΦESDG motif. NV is Norwalk virus (NC_001959), CV is Chiba virus (AB042808), HV is Hawaii virus (U07611), SMV is Snow Mountain virus (AY134748), U201 is Saltama U201 virus (AB039782), and MD145 is MD145 virus (AY032605). (B) Alignment of NV p22 with various cellular and viral proteins that contain an ER export signal. VSV G is the vesicular stomatitis virus glycoprotein, LAP is lysosomal acid phosphatase, VZV GPI is varicella zoster virus glycoprotein I, and CD3γ is a component of the T cell receptor. Adapted from Nishimura and Balch, 1997 [9]. (C) Cells were transfected with the plasmid pCMV-UTR-SEAP expressing GFP, GFP-tagged NV p22, or the indicated mutants within the ER export signal of p22. Secreted SEAP was quantified (see Materials and Methods) as a representative indicator of cellular protein secretion at the indicated time points and was defined by the equation: Secreted SEAP = (SEAP_{extracellular})/(SEAP_{extracellular} + SEAP_{intracellular}) × 100. Data are representative of three independent experiments (n = 3 for each time point; ±SD). (D and E) p22 with the YXΦESDG motif mutated to AXΦASDG (D; E, bottom panels) or wildtype NV p22 (E, top panels) were expressed as GFP fusion proteins. Cells were fixed at 24 hpt and stained with antibody against the cis Golgi marker protein GM130 (D) or the endoplasmic reticulum marker protein calnexin (E) (Alexa 594-conjugated secondary antibody, red fluorescence). Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy. The inset in E is an 8X magnification of the boxed region. doi:10.1371/journal.pone.0013130.g005

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showing that the observed decreases in inhibition of protein secretion were not due to changes in protein expression or stability.

To examine Golgi phenotype in cells expressing the AX- Φ ASDG mutant of p22, we next explored the phenotype of the Golgi in cells expressing this construct. By electron microscopy at 48 hpt and after flow sorting, cells expressing p22(AX Φ ASDG) had wildtype Golgi (data not shown) with intact cisternae present in 26 of 56 (46%) cells, similar to the frequency of intact Golgi in the presence of GFP alone. Consistent with this observation and the inability of p22(AX Φ ASDG) to inhibit SEAP secretion, the Golgi was intact in cells expressing p22(AX Φ ASDG) by immunofluorescence at 24 hpt (Figure 5D). Moreover, the mutant was much more diffuse throughout the cytoplasm of cells with few to no puncta, and exhibited a prominent reticular pattern suggestive of ER localization. To confirm this observation, we next stained cells expressing wildtype p22 or p22(AX Φ ASDG) for the ER marker protein calnexin, the use of which was validated by complete co-localization with protein disulfide isomerase (Figure S3), another ER marker protein.

Wildtype p22 did not co-localize with the ER-marker protein calnexin (Figure 5E), as would be expected for a protein that utilizes an ER export signal [40,41]. In contrast, the AX Φ ASDG construct of p22 exhibited a more diffuse and reticular staining pattern, much of which overlapped with the ER (Figure 5E, inset), suggesting that the mutated residues were either re-localizing the protein to the ER or slowing the trafficking of the protein from the ER. Constructs that had individual mutations within the Y and E residues had intermediate ER localization, and a construct that had both the glutamic and aspartic acid residues mutated to alanine exhibited ER localization and SEAP secretion equivalent to that of the YX Φ ASDG construct (data not shown). These data indicate that the Y and E residues within the conserved YX Φ ESDG motif are necessary for the proper localization of p22 and may constitute a unique arrangement of an ER export signal.

Taken together, these data demonstrate that despite altered trafficking within the secretory pathway, p22(AX Φ ASDG) that was not ER-localized appeared to be retained at the Golgi, reflecting the ability of ER-localized proteins to be non-specifically trafficked from the ER to the Golgi in the absence of a specific export signal [9,10,40]. This indicates that the Y and E residues of the YX Φ ESDG motif together are critical for the cellular localization of p22, as well as inhibition of protein secretion and Golgi disassembly.

Although the conserved YX Φ ESDG motif was clearly critical to the cellular effects exhibited by p22, two aspects of this signal remained unclear: 1) if this motif is indeed a functional ER export signal; and 2) if this motif alone is sufficient to antagonize ER/Golgi trafficking. To answer both of these questions, we next directly tested if the conserved motif within p22 could functionally substitute for a *bona fide* ER export signal. To do this, the well-characterized ER export signal within the vesicular stomatitis virus glycoprotein (VSV G) was replaced with either the conserved motif found within p22 (G/p22), the mutated p22 signal that abrogates the downstream effects of p22(G/AX Φ A), or a construct of VSV G that has the entire ER export signal mutated to all alanines (G/6xA) and has a significantly decreased rate of ER export [40] (Figure 6A). The efficiency of ER export of VSV G or the various chimeric or mutant G proteins was examined by a previously described technique [9,10,40,42] in which newly made protein is metabolically labeled with 35 S-methionine. At the indicated time points, G is purified and digested with EndoH, which only digests G that has not yet reached the Golgi, resulting

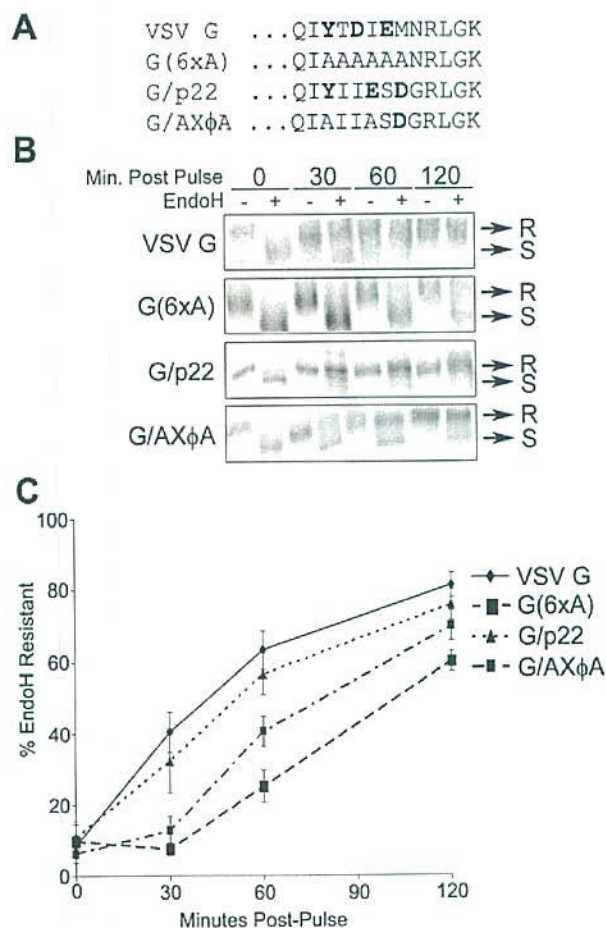


Figure 6. The ER export signal mimic of p22 can substitute for the signal of VSV G. (A) Summary of the sequence of the wildtype, mutant, and chimeric VSV G proteins used. Critical residues of the VSV G ER export signal and homologous regions of p22 are shown in bold. (B) Representative individual samples from EndoH sensitivity assay of VSV G proteins. Wildtype (G), mutant [G(6xA)] and chimeric (G/p22 and G/AX Φ A) VSV G proteins were metabolically labeled with 35 S-Methionine at 24 hours post-transfection and incubated for the indicated period of time, harvested in lysis buffer, immuno-precipitated with monoclonal antibody against the luminal domain of VSV G and digested with endoglycosidase H (EndoH). R = EndoH resistant; S = EndoH sensitive. (C) Cells were transfected with plasmids encoding the indicated constructs of VSV G. At 24 hours post-transfection, cells were labeled with 35 S-Met and at various times post-pulse cells were harvested, immuno-precipitated with antibody against the luminal domain of VSV G and assayed for their sensitivity to EndoH. Data are composite (mean $\pm$ SD) of six individual samples (n=6) for each time point from two independent experiments. doi:10.1371/journal.pone.0013130.g006

in a shift in its apparent molecular weight following resolution by SDS-PAGE (Figure 6B). The percent of G that is resistant to EndoH allows quantitation of the amount of protein that is ER-associated compared to protein that had reached the Golgi, thereby yielding a direct measure of ER export efficiency.

As expected, wildtype VSV G rapidly became resistant to EndoH digestion, whereas G(6xA) exhibited a significant decrease in kinetics of ER export compared to wildtype G [$p < 0.00001$ for all time points except 0 minutes post-pulse (mpps)] (Figure 6C). In contrast, G/p22 demonstrated ER export statistically indistinguishable from wildtype VSV G ($p \geq 0.07$ for all time points except

22

0 mpp), indicating that the conserved motif found within p22 can substitute for the signal found within G and is therefore a functional ER export signal. In contrast, and similar to G(6xA), G/AXΦA had significantly decreased kinetics of ER export ($p \leq 0.001$ for all time points except 0 mpp). The ability of G/AXΦA to ultimately become resistant to EndoH digestion more efficiently than G(6xA) is likely attributable to the presence of the aspartic acid residue on G/AXΦA, which has previously been shown to improve ER export efficiency compared to alanines alone [9,10,40]. In addition, the Golgi was phenotypically intact in cells expressing both wildtype G and G/p22 (data not shown), suggesting that protein trafficking is not inhibited due solely to the presence of the YXΦESDG motif.

Taken together, these data suggest that the motif found within p22 can function for the well-characterized ER export signal found within VSV G and thus this motif constitutes a true ER export signal. For this reason, we henceforth refer to the YXΦESDG motif within p22 as a mimic of an ER export signal, or MERES, motif. In addition, mutation of two residues within the MERES motif that are critical to the function of p22 is sufficient to decrease ER export efficiency. This observation coupled with the inability of the MERES motif alone to induce Golgi disassembly when expressed on VSV G demonstrated that it alone is insufficient to antagonize ER/Golgi trafficking.

p22 targets COPII, but not COPI, trafficking

We lastly sought to determine, first, what is the fate of the cargo in the secretory pathway? And second, does p22 target the anterograde or retrograde pathway of ER/Golgi trafficking? To answer both of these questions, we utilized the SEAP vector described above to monitor the localization of COPI and COPII vesicle marker proteins with respect to secretory pathway cargo, in this case again monitoring SEAP itself as the cargo, and alterations that occur due to the presence of p22.

The 3A protein from several picornaviruses inhibits protein secretion by deregulating COPI vesicle budding from the *cis* Golgi [12,13,43]. To determine if p22 might utilize a similar mechanism of action, the phenotype of COPI vesicle transport in cells expressing p22 was explored by immunofluorescence of the COPI marker protein β -COP. In the presence of GFP alone, SEAP was present only in an intact Golgi, where COPI puncta are prominently, though not exclusively, localized (Figure 7A). As expected, SEAP was prominently retained in cells expressing both PV 3A (Figure 7B) and NV p22 (Figure 7C), although with different phenotypic localizations. In 3A-expressing cells, COPI puncta were diffuse and unapparent, and SEAP was retained in diffuse, minimally punctate cellular structures reminiscent of Golgi that has been redistributed into the ER due to antagonism of trafficking by 3A, as has been reported previously [12,34,44]. In contrast, COPI puncta in cells expressing p22 were apparent but re-localized widely throughout the cytoplasm, demonstrating a failure of these vesicles to properly localize and/or traffic within cells. SEAP in p22-positive cells was present both in discrete punctate vesicles that did not co-localize with COPI puncta, and also in peri-Golgi clusters that did co-localize with COPI puncta. This suggested that SEAP, and therefore cellular cargo, was being retained in non-COPI puncta and at a *cis* Golgi site due to the expression of p22. This suggested that the retrograde, Golgi-to-ER arm of the secretory pathway was intact, but an aspect of the forward pathway was non-functional in p22-expressing cells. These data demonstrate that, in contrast to 3A, p22 does not specifically target COPI trafficking, but does induce cellular cargo retention between the ER and Golgi.

Due to these observations and because ER export signals promote the rapid and direct uptake of cargo into COPII-coated vesicles, which are necessary for ER-to-Golgi protein trafficking, we hypothesized that p22 is acting on the forward, ER-to-Golgi trafficking pathway to inhibit COPII vesicle budding or trafficking to the Golgi to thereby induce Golgi disassembly and inhibit protein secretion. To test this hypothesis, we again examined the specific sub-cellular localization of the retained SEAP that was present in discrete cytoplasmic puncta. Under the same dicistronic SEAP expression system, in the presence of GFP alone SEAP was again localized exclusively in a phenotypically intact Golgi with COPII puncta immediately surrounding it, although with a near complete lack of co-localization with COPII puncta (Figure 8A). In contrast, in the presence of p22 SEAP was localized in peri-nuclear puncta (Figure 8B), similar to the phenotype of a disassembled Golgi previously described (Figure 2A). Both SEAP and p22 also co-localized with COPII puncta that were also prominently re-localized to the same presumably peri-Golgi structures, although there was some diffuse, likely ER-localized, COPII staining that did not localize with either p22 or SEAP. Additionally, these discrete p22-positive puncta co-localized with SEAP and COPII vesicles (Figure 8B, inset). This suggested that p22 and SEAP are retained with COPII puncta that have properly budded from the ER, but have been mislocalized and did not properly traffic into the Golgi. Due to the apparent size of the p22/SEAP/COPII puncta observed by immuno-fluorescence, it is tempting to speculate that the cellular vesicles with cargo inside them that were observed by EM (Figure 3B) may be enlarged COPII puncta that were mislocalized within cells. When the AXΦA mutant of p22 was expressed, SEAP and COPII puncta both returned to a wild type distribution (Figure 8C) that was phenotypically indistinguishable from cells expressing GFP alone. This demonstrated that, in the presence of p22 and dependent upon the MERES motif, both COPII puncta and their cargo are mislocalized, suggestive of a failure of vesicles to either traffic to or fuse with the Golgi apparatus.

Discussion

We have described for the first time a novel function for the Norwalk virus nonstructural protein p22. The major new findings of this study can be summarized as follows: 1) independent expression of p22 disassembles the Golgi, which also occurs during NV replication, and inhibits cellular protein secretion; 2) subcellular localization of p22 depends on a motif that mimics a cellular ER export signal in both sequence and function, which we have named a MERES motif; and 3) p22 depends on the MERES motif likely to antagonize COPII vesicle trafficking, resulting in Golgi disassembly and an inhibition of cellular protein secretion. Due to the observed trafficking of p22, we propose that p22 is initially localized to the membranes of the secretory pathway via amino acids 103–148, which contain a membrane association domain. The MERES motif then mediates the uptake of p22 onto COPII vesicles, which mislocalize within cells and inhibit, by an as-of-yet undetermined mechanism, proper trafficking to or fusion with the Golgi. Restricting the proper flow of COPII vesicles then induces disassembly of the Golgi and ultimately results in an inhibition of cellular protein secretion.

The MERES motif found within p22 is somewhat unconventional for an ER export signal in that p22 does not encode a signal sequence to mediate ER import, is not glycosylated, and is not exclusively ER localized at any point during expression (Figure 4D and data not shown). Moreover, it has several unique features compared to other known di-acidic ER export signals. First,

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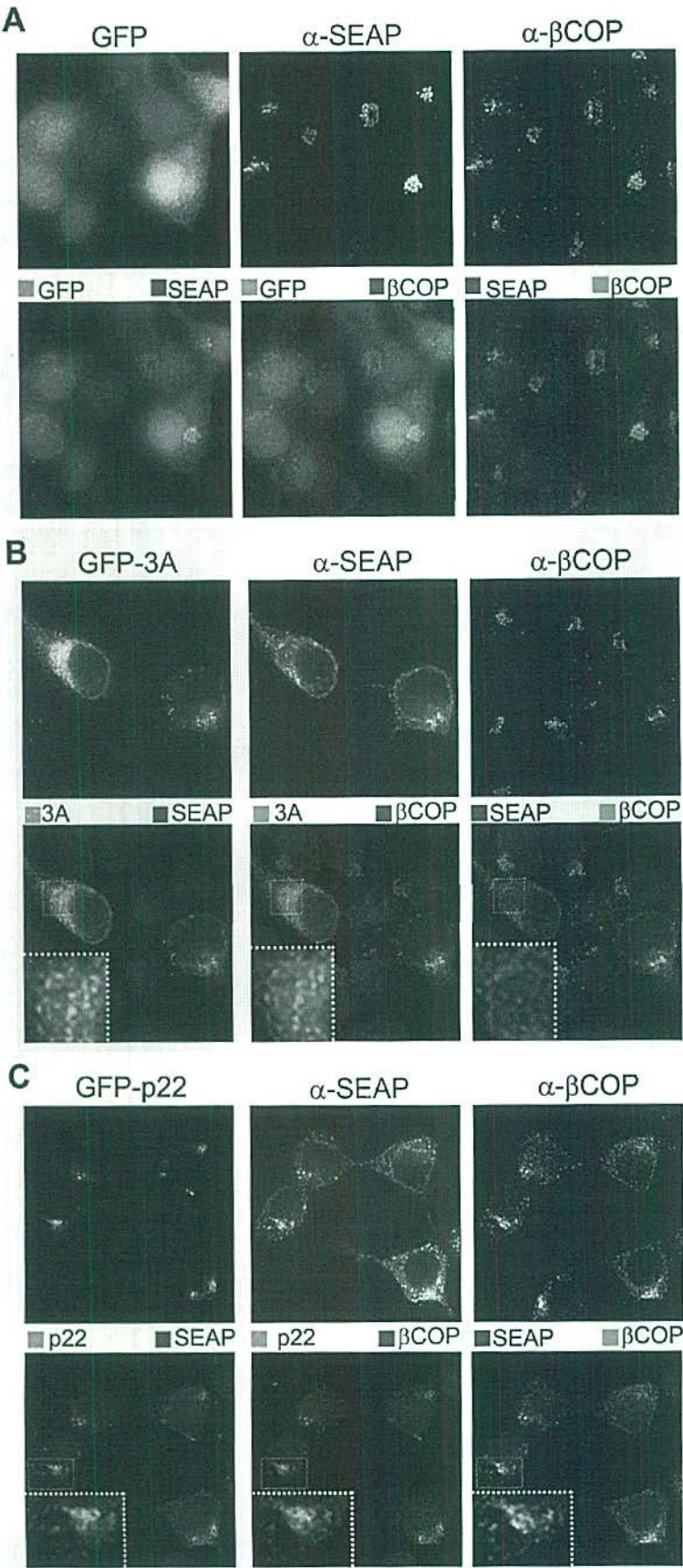


Figure 7. SEAP is differentially retained in the presence of PV 3A and NV p22. Cells expressing GFP alone (A), or GFP-tagged 3A (B) or NV p22 (C) were fixed at 24 hpt and immuno-stained with antibody against SEAP (Alexa 594-conjugated secondary antibody) and the COPII marker protein β -COP (Alexa 647-conjugated secondary antibody), stained with DAPI (blue fluorescence), and imaged by deconvolution microscopy. Channels were pseudo-colored as indicated for merged images. Insets in B and C represent a 6X zoom of the boxed region. doi:10.1371/journal.pone.0013130.g007

instead of the tyrosine residue being located between the MAD and the acidic residues, it is located N terminal to the acidic residues and MAD. Second, instead of encoding two amino acids between the Y and Φ residues, p22 encodes only a single residue. Third, most, though not all, ER export signals are located towards the extreme C terminus of proteins, whereas the signal found within p22 is located in the middle of the protein. The human asialoglycoprotein receptor H1 subunit is the only other protein described to have this arrangement of a di-acidic ER export signal [9]. Additionally, we have no data to support any portion of p22 being present inside the lumen of the ER and instead p22 appears to be a peripheral membrane protein based on sodium carbonate extraction (data not shown). For these reasons, the YX Φ ESDG motif found within p22, though similar in sequence and function to a traditional di-acidic ER export signal, may in reality simply mimic the function of these signals to exploit the cellular machinery responsible for protein secretion in order to recruit p22 to COPII vesicles. This lends support to the nomenclature of this motif being a mimic of an ER export signal, as it shares many of the features and effects of a traditional ER export signal, but ultimately has a unique composition and function.

Furthermore, p22 seems to depend on the MERES motif to function as a secretory pathway inhibitor; however this motif alone is not responsible for antagonism, as replacement of the ER export signal from VSV G with the MERES motif functionally restored ER export efficiency without any obvious impediment. In support of this, none of the deletion mutants of p22 induced Golgi disassembly, although amino acids 50–148 were sufficient to mediate Golgi localization. Similarly, these same residues were sufficient to mediate COPII localization, but do not lead to the mislocalization of COPII puncta (data not shown) that was observed for full length p22. Taken together, these data suggest that the MERES motif of p22 alone is necessary but not sufficient to inhibit the secretory pathway. A second as-of-yet uncharacterized factor, contained within or dependent on the N and/or C termini based on deletion mutant experimentation, is required as a subsequent step in secretory pathway antagonism. This idea is substantiated by chimeric G/p22 properly trafficking to the Golgi and becoming resistant to Endo H digestion with kinetics equivalent to wildtype G. Possibilities for the function of this second factor are many, including binding to and/or disrupting a cellular tethering protein [45,46] or a member of the p24 family that covers COPII vesicles [47], or antagonizing SNARE-mediated vesicle fusion by interaction with a regulatory protein such as VAP-A/B [48], all of which are critical components of ER/Golgi trafficking. This latter possibility is especially attractive, as VAP-B has previously been demonstrated to be a binding partner for another Norwalk virus nonstructural protein [49], and thus may be an over-arching target of the secretory pathway by noroviruses. Both of these possibilities are currently under investigation as potential targets for p22 and as scaffolding factors upon which noroviruses may anchor their genome replication.

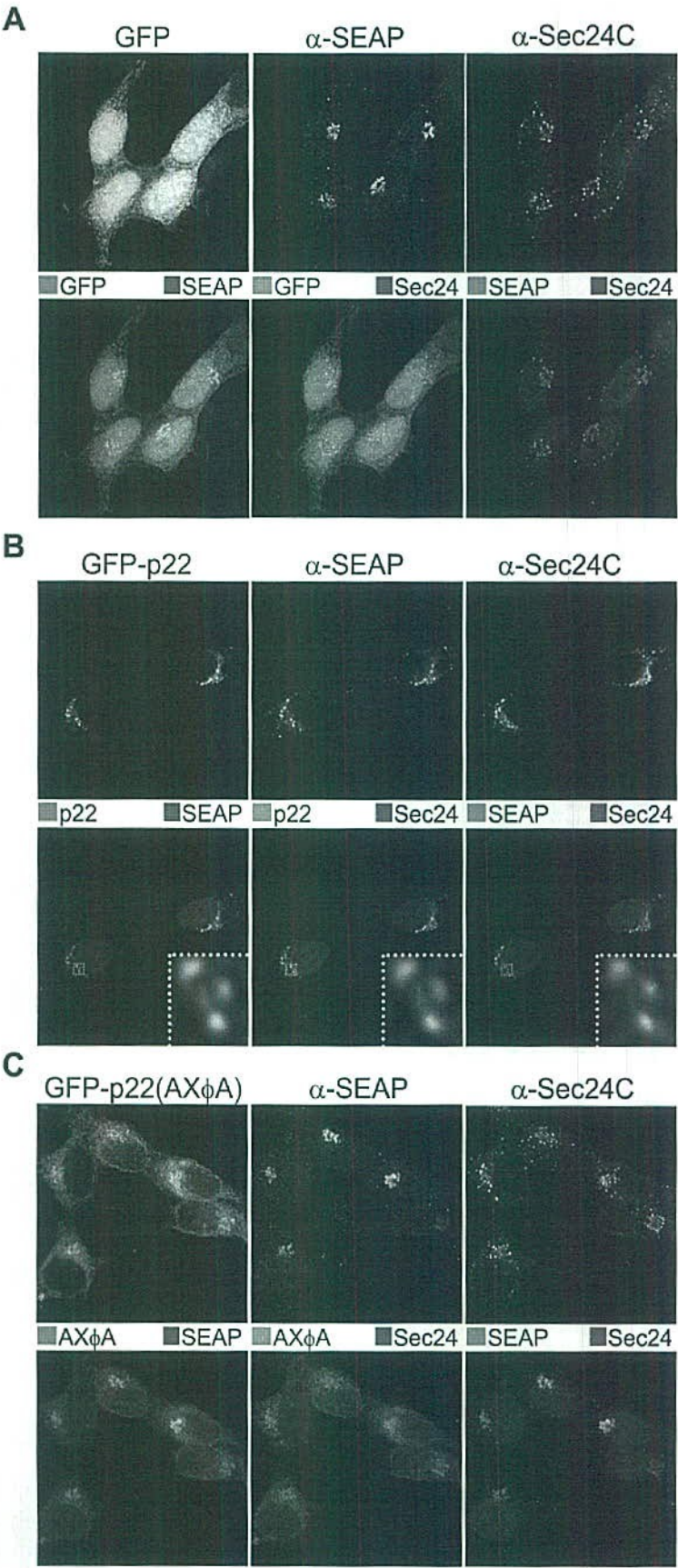
The data presented in this study support, but do not prove, that p22 targets COPII vesicle trafficking; this is further complicated by the examination of cells by immunofluorescence primarily at 24 hpt, which may not reflect steady-state localization. Proving this mechanism will require demonstrating a specific binding to or inhibition of the trafficking of COPII vesicles using *in vitro* assays.

Unfortunately, these studies have not been possible due to the inability of producing purified p22 for such studies as well as the lack of an antibody to p22 that allows for immunoprecipitation assays. Therefore, until a direct interaction is demonstrated, it remains possible that p22 could target a non-COPII aspect of the secretory pathway to mediate inhibition. p22 does not activate Arf1 or Sar1 (data not shown), making targeting of the formation of COPI vesicles in the same manner as PV and CVB3 3A, or COPII vesicles by a unique approach, unlikely. A *trans* Golgi mechanism of action seems similarly unlikely based on lack of localization of wildtype p22 with the *trans* Golgi marker protein golgin 97 (Figure S2); the same is also true for possible targeting of endosomes by p22 (data not shown). Thus, although there are many possible alternative explanations for the observed effects of p22, specific targeting and mislocalization of COPII vesicles is at present the most likely explanation.

Although no other cellular or microbial protein to date has been described to use the arrangement of a MERES motif that p22 employs to inhibit the secretory pathway, several previously characterized secretory pathway antagonists have potential ER export signals or mimics thereof. The *Escherichia coli* protein NleA inhibits COPII-dependent export from the ER by direct interaction with Sec24 [50], and the cellular proteins STAM-1 and -2, which are involved in the signaling of growth factors and cytokines, regulate Golgi architecture by interaction the Sec13/31 COPII cage components [51]. Examination of the primary amino acid sequence of NleA and STAM-1/2 revealed motifs similar to a di-acidic ER export signal (unpublished observation). If further studies determine these motifs directly contribute to either the cellular localization or, for STAM-1/2, proper ER export, this would provide support for the idea that ER export signals or their mimics can be used not only to facilitate ER export, but also to promote interaction with COPII vesicles to mediate specific antagonism of the secretory pathway.

Both similarities and differences were noted between p22 and the picornavirus 3A protein. Both proteins localize to membranes via an amphipathic alpha helix, and both inhibit ER-to-Golgi trafficking to decrease cellular protein secretion. However, the mechanism of this shutoff appears to be quite distinct between Norwalk virus and picornaviruses. Inhibition of protein secretion and Golgi disassembly are in some cases separable and distinct, as is the case for PV infection [44], whereas in other cases one will follow the other, for example during cell division [31,52]. There were also clear ultrastructural similarities between cells expressing p22 and 3A in inducing the accumulation of free membranes, double-membrane vesicles and vacuoles [12,34], although cells expressing p22 did not exhibit the swelling of the ER reported for PV 3A [34] or the crystalloid ER patterns seen after expression of the hepatitis A virus 2C and 2BC proteins that also induce significant membrane rearrangements [53]. This further supports the similarity of p22 and 3A in secretory pathway antagonism, but through different arms of this pathway. NV p22 therefore may be more similar in the cellular effects of the hepatitis C virus NS4A/B protein, which, though less studied than PV 3A, antagonizes ER-to-Golgi trafficking, and induces the accumulation of “membranous webs,” vacuoles and double-membrane vesicles, but not ER swelling [54].

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Figure 8. Secretory pathway cargo is retained in COPII vesicles in the presence of p22, but not p22(AXΦA). Cells expressing GFP alone (A), or GFP-tagged NV p22 (B) or p22(AXΦA) (C) were fixed at 24 hpt and immuno-stained with antibody against SEAP (Alexa 594-conjugated secondary antibody) and the COPII marker protein Sec24C (Alexa 647-conjugated secondary antibody), stained with DAPI (blue fluorescence), and imaged by deconvolution microscopy. Channels were pseudo-colored as indicated for merged images. Inset in B represents an 8X zoom of the boxed region.
doi:10.1371/journal.pone.0013130.g008

Although all the effects of NV infection on the secretory pathway have not yet been explored, the results presented here demonstrate that, like PV 3A, direct antagonism of the secretory pathway is a cause, not an effect, of Golgi disassembly by p22. However, whereas at least two picornavirus 3A proteins interact with the cellular protein GBF-1 to inhibit COPI vesicle budding [13], NV p22 instead appears to target COPII vesicles with dependence upon a motif that is absent from picornavirus 3A proteins, suggesting that these two proteins take different approaches to the same ultimate outcome. It therefore appears that, like several picornaviruses [28,29], noroviruses encode two proteins, p22 and p48 (this study and [27]), with redundant functions of antagonizing the secretory pathway.

FCV p30, a homologue of p22, lacks an ER export signal and localizes exclusively to the ER when independently expressed ([26] and our unpublished observation). This is in line with the inability of BFA to inhibit FCV replication [24], as it does several picornaviruses, and further supports the notion that caliciviruses utilize a different architecture of cellular machinery for replication than do picornaviruses. A recent study has shown that MNV is also resistant to the cellular effects of BFA and does not have an effect on gross Golgi morphology [55]. It will therefore be interesting to determine the roles of p22 homologues from MNV and FCV in possible antagonism of ER/Golgi trafficking, as this would shed much light on similarities and differences between animal and human caliciviruses.

The biological significance of antagonism of the secretory pathway by p22 remains to be understood. This may facilitate viral pathogenesis rather than replication in a manner similar to the effect of the picornavirus 3A proteins that inhibit the immune response to virus-infected cells [14–16], ultimately leading to a more pathogenic infection [12]. Future study of the immune response to norovirus infection should consider secretory pathway antagonism by p22, as this protein may be key in deactivating interferon (IFN) and/or cytokine signaling following infection. Additionally, analysis of the cellular response to NV infection has demonstrated that NV is sensitive to IFN when exogenously added to cells replicating the NV genome [56,57]; however, NV does not induce the IFN pathway or IRF3 activation in Huh7 cells that support a single round of virus replication [58]. Although p22 may be contributing to a reduction in IFN release from cells, the possibility that p22 has additional inhibitory effects on the IFN pathway remains to be explored.

Since viruses utilize cellular processes and machinery for replication, understanding the mechanisms by which viruses parasitize the cell will both increase our understanding of these pathways and aid in the design of effective anti-viral countermeasures. The human norovirus nonstructural protein p22 encodes a novel and well-conserved motif that mimics a traditional di-acidic ER export signal. Instead of increasing the rate of protein trafficking in the secretory pathway, as is the normal function of these signals, the ER export signal mimic allows p22 to gain access to the secretory pathway, induce Golgi disassembly and inhibit cellular protein secretion. This is the first instance in which a pathogen has been described to use a motif similar to an ER export signal to ultimately inhibit cellular protein secretion. This motif constitutes a new target for the design of anti-viral drugs

against noroviruses, as it is necessary for the antagonistic activity of p22 and is highly conserved in human noroviruses.

Materials and Methods

Vectors and cell lines

All genes were cloned using Gateway technology (Invitrogen) and expressed in pcDNA-DEST53 after sequence verification. Mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). HEK-293T and Huh7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. 293T cells were grown on plastic or coverslips coated with 10 µg/ml poly-D-lysine (Sigma). Transfection was carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. cDNA encoding poliovirus 3A protein was obtained from Richard Lloyd of Baylor College of Medicine.

Transfection of NV RNA

NV RNA was purified from volunteer stool samples and transformed into Huh7 cells as described previously [30]. Briefly, virus was purified from volunteer stool samples by sucrose cushion and cesium chloride gradient extraction, following which viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Five hundred nanograms of purified viral RNA were then transfected into cells, which were then cultured for the indicated period of time.

Immunofluorescence and antibodies

Cells were grown on cover slips and, following the indicated treatments, washed in PBS and subsequently fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PBS and permeabilized with 0.5% Triton X-100 in 0.1 M PBS. Cells were then washed in PBS and blocked with 1% bovine serum albumin (Sigma, St. Louis, MO) for 1 hour at 37°C, incubated overnight at 4°C with primary antibody in 0.1 M PBS, 1% BSA. Anti-human golgin-97 mouse monoclonal antibody was obtained from Molecular Probes (Invitrogen). GM130 mouse monoclonal antibody was obtained from BD Transduction Laboratories (San Jose, CA). Calnexin mouse monoclonal antibody and rabbit polyclonal antibody against β-COP were obtained from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody against Sec24C was the generous gift of Bill Balch of Scripps Research Institute and has been previously described [59]. Rabbit polyclonal antibody against VP1 was also previously described [60]. Mouse monoclonal antibody 8B6 against placental alkaline phosphatase was obtained from AbCam (Cambridge, MA). Rabbit polyclonal antibody against protein disulfide isomerase (H-160) was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). After incubation with primary antibody, cover slips were washed and incubated at room temperature for one hour with the corresponding AlexaFluor 488-, 594-, or 647-conjugated secondary antibodies (Invitrogen). Nuclei were stained with 300 nM DAPI (Invitrogen) at room temperature for 5 minutes. Cells were then washed and mounted using the ProLong Gold antifade reagent (Invitrogen). All images shown are representative of at least three independent experiments and the direct observation of

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no less than 50 cells with GFP expression levels in approximately the middle 70th percentile.

Fluorescence deconvolution microscopy

Deconvolution microscopy was performed with a Zeiss AxioVert S100 TV microscope and a DeltaVision restoration microscopy system (Applied Precision, Inc.) and imaged using either a 63X objective lens (1.40 NA). A *z* series of focal planes was digitally imaged and deconvolved with the Delta-Vision constrained iterative algorithm to generate high-resolution images, from which Quick Projections were obtained. Brightness and contrast levels were adjusted appropriately in Adobe Photoshop version CS2.

Flow-coupled electron microscopy

293T cells were transfected with the indicated plasmids and at 24 hpt were harvested in 0.1 M PBS containing 1% BSA. Cells were then flow sorted for GFP on a BD SORP FACSaria II flow cytometric cell sorter with elution into 10% DMEM. Cells were then centrifuged at 500×g for 10 minutes, suspended in 10% DMEM and plated onto plastic dishes coated with 10 µg/ml poly-D lysine. Twenty-four hours later, and therefore 48 hpt, all cells were validated to be GFP positive under an epifluorescence microscope. Cells were then rinsed once in 0.1 M PBS and fixed in Karnovsky's Fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer +2 mM CaCl₂, pH 7.4) for 1 hour on ice. After being held overnight at 4°C in weak fix (1 part Karnovsky's Fixative: 10 parts 0.1 M cacodylate buffer +2 mM CaCl₂), the cells were rinsed 3 times in 0.1 M cacodylate buffer +2 mM CaCl₂, then post-fixed in 1% OsO₄ in 0.1 M cacodylate at 4°C. After 3 rinses in cacodylate buffer, cells were dehydrated in a gradient series of ethanol from 30–50%, en bloc stained with saturated uranyl acetate in 50% ethanol for 1 hour, carried through to 100% ethanol, then infiltrated in 1 part 100% ethanol: 1 part Spurr's Low Viscosity Resin overnight at room temperature. The rest of the infiltration was performed the next day, through 3 changes of pure resin. A small amount of pure resin was placed in the bottom of thoroughly drained plates, and the plated cells and resin were cured at 60°C for 3 days. Sections were cut at 70–80 nm using a Diatome diamond knife and an RMC MT6000-XL ultramicrotome. Sections were then collected on 100–150 mesh copper grids, counter-stained with Reynold's lead citrate and viewed on a Hitachi H-7500 transmission electron microscope. Image brightness and contrast were adjusted appropriately in Adobe Photoshop version CS2.

Cytosolic and membrane fractionation

Transfected 293T cells were suspended in 0.5 ml of homogenization buffer [200 mM HEPES (pH 7.5), 5 mM sodium pyrophosphate, 5 mM EGTA, 1 mM MgCl₂, 1 mM sodium orthovanadate, 50 µM leupeptin, 200 µM PMSF, 1 µM pepstatin A], followed by sonication and centrifugation at 100,000×g for one hour at 4°C, and the resulting supernatant was the cytosolic fraction. Pellets were resuspended in 0.5 ml extraction buffer [20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM NaF, 1 mM sodium orthovanadate, 50 µM leupeptin, 200 µM PMSF, 1 µM pepstatin A], followed by incubation with rotation at 4°C for one hour and centrifuged at 100,000×g for 1 hour and the supernatant was collected as the membrane fraction. Fractions were then precipitated by addition of trichloroacetic acid to 10% and centrifugation at 16,000×g for 5 minutes. The pellet was then washed in acetone, pelleted, and resuspended in 50 µl of 5X SDS-PAGE sample buffer. Lysate aliquots were boiled for 3

minutes and analyzed by electrophoresis on 12.5% SDS-PAGE gels. Separated proteins were transferred from the gel to nitrocellulose membrane (Amersham Biosciences) and membranes were blocked in 5% Blotto (5% fat-free Carnation milk in 0.01 M PBS) and incubated with a mouse anti-GFP monoclonal antibody (Clontech, Mountain View, CA) in 0.5% Blotto overnight at RT. Primary antibody was removed and membranes were washed three times with 0.5% Blotto. Horseradish peroxidase-conjugated secondary goat anti-mouse immunoglobulin G antibody (Sigma-Aldrich) was incubated with the membranes for ~2 h at RT and subsequently washed three times with 0.5% Blotto. Membranes were developed with SuperSignal sensitivity substrate (Pierce).

SEAP assay

The plasmid pCMV-UTR-SEAP was obtained from J. Lindsay Whitton (Scripps Research Institute) [28] and was made into a Gateway-competent vector using the Gateway Vector Conversion System (Invitrogen) at the *NotI* site of the vector. 293T cells were grown to ~60% confluence in 24 well plates and transfected. Two hours before the indicated time points, cells were washed 2 times in 0.01 M PBS and the media was replaced with 400 µl of fresh media. At the indicated time points, the media containing "extracellular SEAP" was removed. At this same time point, "intracellular SEAP" values were obtained by washing cells once in cold 0.01 M PBS and adding 400 µl of media containing 0.5% Triton X-100 to lyse cells and solubilize any SEAP that had been retained in cells, and then incubating cells at room temperature for ~5 minutes. Lysed cells were then collected and spun at 13,000×g for one minute to remove cellular debris. Enzymatic SEAP activity in "intracellular" and "extracellular" fractions was assayed with the PhosphaLight TM System (Applied Biosystems), and overall Secreted SEAP was calculated with the equation: Secreted SEAP = (SEAP_{extracellular} / (SEAP_{extracellular} + SEAP_{intracellular})) × 100 [28].

EndoH Sensitivity Assay

Sensitivity of VSV G and chimeras to digestion with endoglycosidase H was carried out as previously described [9,40,42] with minor modifications. VSV G or chimeras were expressed in 293T cells from the plasmid pMD2.G. Twenty-four hours post-transfection, cells were washed 3X in Cys/Met-free DMEM and incubated at 37°C for 15 minutes. Cells were then labeled with 200 µCi/ml ³⁵S-Met (Amersham BioSciences) for 15 minutes at 37°C, washed 3X in DMEM containing 10% FBS, and 400 µl of DMEM containing 10% FBS was added to cells, which were then incubated at 37°C. At the indicated time post-pulse, cells were washed 1X in cold PBS and harvested in VSV G Lysis Buffer (50 mM Tris pH 8, 62.5 mM EDTA, 1% NP-40, 0.4% deoxycholate, 50 µM leupeptin, 200 µM PMSF, 1 µM pepstatin A). Supernatants were cleared with 5 µl of Protein A Magnetic Beads (Invitrogen) and incubated overnight with monoclonal antibody against the luminal domain of VSV G (clone 8G5) [42], generously provided by Bill Balch, Scripps Research Institute. Immuno-complexes were collected by magnetic capture and washed 2X in VSV G Lysis Buffer, resuspended in 40 µl of 2.5X SDS-PAGE Sample Buffer and boiled for 5 minutes. Half of the IP was digested with 1 µl of endoglycosidase H (EndoH) (1 U/200 µl, Roche) at 37°C for 1 hour. EndoH treated and non-treated samples were then run on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and bands were quantitated following scanning on a Typhoon Trio Variable Mode Imager (GE Healthcare) and ImageQuant 5.1 to quantitate band intensity.

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Statistical analysis

Two-tailed, unpaired Student's *t*-tests assuming unequal variance were used to determine statistical significance; *p* values are indicated where appropriate.

Supporting Information

Figure S1 NV p22 initially localizes to and induces disruption of the trans Golgi. Cells expressing GFP or GFP-tagged Norwalk virus (NV) p22 were immuno-stained for the trans Golgi marker protein golgin-97 (Alexa 594-conjugated secondary antibody, red fluorescence) at the indicated times post-transfection. Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy.

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Figure S2 Equivalent amounts of protein are made during SEAP analysis of p22 constructs containing alanine mutations within the predicted ER export signal. Proteins in lysates from the 36 hpt intracellular fraction of cells utilized in the indicated SEAP assay were run on a 4–20% SDS-PAGE gel and detected with monoclonal antibody against either GFP or actin by western blot.

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Figure S3 Calnexin is an appropriate marker of the endoplasmic reticulum in 293T cells. Non-transfected 293T cells were fixed and immuno-stained for the ER marker proteins calnexin and protein disulfide isomerase with mono- (red fluorescence, Alexa594-conjugated secondary antibody) and poly-clonal antibody (green fluorescence, Alexa488-conjugated secondary antibody), respectively. Nuclei were stained with DAPI (blue fluorescence) and cells were imaged by deconvolution microscopy.

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Author Contributions

Conceived and designed the experiments: TMS SG KK SEC MKE. Performed the experiments: TMS SG. Analyzed the data: TMS SEC MKE. Contributed reagents/materials/analysis tools: KK. Wrote the paper: TMS MKE.

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Enteropathogenic *E. coli* attenuates secretagogue-induced net intestinal ion transport but not Cl^- secretion

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Hecht, Gail, and Athanasia Koutsouris. Enteropathogenic *E. coli* attenuates secretagogue-induced net intestinal ion transport but not Cl^- secretion. *Am. J. Physiol.* 276 (Gastrointest. Liver Physiol. 39): G781–G788, 1999.—Enteric bacterial pathogens often increase intestinal Cl^- secretion. Enteropathogenic *Escherichia coli* (EPEC) does not stimulate active ion secretion. In fact, EPEC infection decreases net ion transport in response to classic secretagogues. This has been presumed to reflect diminished Cl^- secretion. The aim of this study was to investigate the influence of EPEC infection on specific intestinal epithelial ion transport processes. T84 cell monolayers infected with EPEC were used for these studies. EPEC infection significantly decreased short-circuit current (I_{sc}) in response to carbachol and forskolin, yet ^{125}I efflux studies revealed no difference in Cl^- channel activity. There was also no alteration in basolateral K^+ channel or $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport activity. Furthermore, net $^{36}\text{Cl}^-$ flux was not decreased by EPEC. No alterations in either K^+ or Na^+ transport could be demonstrated. Instead, removal of basolateral bicarbonate from uninfected monolayers yielded an I_{sc} response approximating that observed with EPEC infection, whereas bicarbonate removal from EPEC-infected monolayers further diminished I_{sc} . These studies suggest that the reduction in stimulated I_{sc} is not secondary to diminished Cl^- secretion. Alternatively, bicarbonate-dependent transport processes appear to be perturbed.

infectious diarrhea; enteric pathogens; bicarbonate

THE PARADIGM TYPICALLY associated with diarrhea that results from infection by an enteric pathogen is one of Cl^- secretion or, somewhat less commonly, inhibition of Na^+ and Cl^- absorption. As more effort has been invested into studying the interactions between infectious agents and their target host cells, previously unrecognized mechanisms of pathogenesis have been defined. For example, it is now clear that several enteric bacterial pathogens and/or their toxins alter tight junction permeability of intestinal epithelia (9). Some, such as toxins A and B of *Clostridium difficile*, appear to induce this physiological function by affecting the actin cytoskeleton and specific tight junction proteins via inactivation of Rho (10, 11, 21). Others may

directly affect tight junction structure, such as zonula occludens toxin of *Vibrio cholera* (8). Still others, such as enteropathogenic *Escherichia coli* (EPEC), alter tight junction permeability by activating specific signaling pathways (24). EPEC attachment to its host target cell induces the phosphorylation of myosin light chain, thereby stimulating contraction of the perijunctional actomyosin ring and opening tight junctions (27). Perturbation of tight junction barrier function may interfere with vectorial transport by allowing back diffusion and equilibration of electrochemical gradients to occur. This isolated alteration in intestinal epithelial physiology, however, is unlikely to be sufficient to account for the resultant diarrhea associated with EPEC.

EPEC is a most interesting pathogen as it is neither invasive nor toxigenic. Yet somehow by intimately attaching to its host cell, it activates a number of signal transduction pathways that then presumably translate into alterations in intestinal epithelial function, in turn resulting in diarrhea. Interestingly, we and others have previously reported (23, 26) that cultured intestinal epithelial T84 cell monolayers infected with EPEC exhibit an attenuation in net ion transport, measured as short-circuit current (I_{sc}), in response to classic secretagogues such as forskolin and carbachol. This observation, presumed to reflect decreased Cl^- secretion, has not been examined in detail. The aim of this study was to investigate the effects of EPEC on intestinal epithelial transport processes with specific attention to Cl^- secretion. For these investigations, we have employed the well-characterized human intestinal epithelial cell line, T84, and a commonly used clinical isolate of EPEC, strain E2348/69.

METHODS

Culture of T84 cells. T84 cells were kindly provided by Dr. Kim Barrett (University of California, San Diego, CA). Passages 25–40 were used for these studies. Cells were cultured as previously described (18) in a 1:1 mixture of Ham's F-12 and DMEM supplemented with 6% newborn calf serum.

Bacterial cultures. EPEC strain E2348/69, a gift from Dr. James Kaper (Center for Vaccine Development, University of Maryland, Baltimore, MD), was initially grown overnight in Luria-Bertani broth. On the day of experimentation, 30 μl of EPEC culture were transferred to 1 ml of serum- and antibiotic-free T84 cell medium and grown to early or midlog growth phase. T84 cell monolayers were then infected with 2×10^7 colony-forming units (cfu) of EPEC for 1 h. Nonadher-

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ent bacteria were then removed by washing, and incubation proceeded for another 1–2 h. EPEC strain UMD864, an *espB* deletion mutant, was provided by Dr. Michael Donnenberg (Section of Infectious Diseases, University of Maryland, Baltimore, MD).

Electrophysiological studies. A simplified apparatus for measuring electrophysiological parameters previously described by Madara et al. (16) was used for these studies. I_{sc} in response to carbachol (10^{-4} M) or forskolin (10^{-6} M) was determined. Resistance was calculated using Ohm's law ($V = IR$, where V is voltage, I is current, and R is resistance) by determining voltage produced in response to the passage of 25 μ A of current. All experiments were performed in Ringer solution containing (in mM) 114 NaCl, 5 KCl, 1.65 Na_2HPO_4 , 0.3 NaH_2PO_4 , 25 NaHCO_3 , 1.1 MgSO_4 , 1.25 CaCl₂, and 5 glucose unless indicated otherwise. For select experiments, specific ion-free buffers were used. Na^+ -free buffer contained (in mM) 116 choline chloride, 5 KCl, 1.65 K_2HPO_4 , 0.3 KH_2PO_4 , 25 choline HCO_3^- , 1.1 MgSO_4 , 1.25 CaCl_2 , and 10 glucose. HEPES-phosphate-buffered Ringer solution (HPBR) was used for HCO_3^- -free studies and contained the following (in mM): 135 NaCl, 5 KCl, 3.33 NaH_2PO_4 , 0.83 Na_2HPO_4 , 1 CaCl_2 , 1 MgCl_2 , 5 HEPES, and 10 glucose.

Experiments examining the effect of Na^+ or HCO_3^- removal on secretagogue-stimulated I_{sc} were performed as follows: T84 monolayers were infected with EPEC for 1 h, after which nonadherent organisms were removed by washing. Incubation was continued for an additional 1–2 h. Select buffers were added to either the apical or basolateral reservoir as indicated and incubated for 1 h. I_{sc} was then measured in response to secretagogues.

^{125}I and ^{86}Rb efflux studies. T84 cell monolayers were grown to confluence on collagen-coated permeable supports (0.4 μ m pore size Transwells; Costar, Cambridge, MA). Control and EPEC-infected monolayers were loaded with 2 μ Ci of either ^{125}I or ^{86}Rb by incubating for 3 h. Monolayers were then rapidly washed four times with HPBR, and a previously described "sample/replace" technique (25) was used to determine the rate constants of ^{125}I and ^{86}Rb efflux to estimate the secretagogue-stimulated activation of Cl^- and K^+ channels, respectively. Before addition of secretagogue, four baseline samples were obtained. Samples were collected every 2 min following activation with secretagogue. Residual intracellular radioactivity was determined following extraction with 1 ml of 0.1 N NaOH. Samples were counted in a scintillation counter. The efflux rate constant was calculated as $[\ln(R_2) - \ln(R_1)]/(t_2 - t_1)$, where R_2 and R_1 are the percentages of radioactivity remaining in the monolayer at times t_2 and t_1 (25).

^{86}Rb uptake. T84 monolayers grown on permeable supports were treated or not with forskolin in the presence and absence of bumetanide (20 μ M for 20 min). HPBR containing 2 μ Ci/ml of ^{86}Rb was then added to the basolateral reservoir of monolayers, and uptake was allowed to proceed over 3 min. Termination of uptake was accomplished by immersing the monolayers in ice-cold buffer composed of 100 mM MgCl_2 and 10 mM Tris·HCl, pH 7.5. Radioactivity was extracted from monolayers with 0.1 N NaOH and counted in a scintillation counter. Protein concentration was determined by the Bradford assay. Na^+ - K^+ -2 Cl^- cotransporter activity was defined as the bumetanide-sensitive portion of ^{86}Rb uptake.

Ussing chamber studies. T84 cell monolayers were grown on 2-cm² collagen-coated polycarbonate filters glued onto lexan rings as previously described (2). Both uninfected control monolayers and EPEC-infected monolayers were used for these studies. Infected monolayers were incubated with EPEC for 3 h before insertion into modified Ussing chambers. Resistances were matched to within 15% for all studies.

Unidirectional fluxes of $^{36}\text{Cl}^-$ were performed utilizing either Ringer solution or HCO_3^- -free HPBR. After a 20-min equilibration period, 2 μ Ci/ml of $^{36}\text{Cl}^-$ (Amersham, Arlington Heights, IL) was added to either the mucosal or serosal reservoir (5 ml each). Four baseline samples were collected before basal stimulation with forskolin. Samples (1 ml) were then collected from the "cold" reservoir at 2-min intervals for 12 min. Sample volume was replaced with the appropriate solution. At the end of the experiment, 250 μ l were collected from the "hot" reservoir so that flux rates could be calculated as previously described (11). Net Cl^- flux is calculated as the mucosal-to-serosal flux rate minus the serosal-mucosal flux rate. Unidirectional fluxes of $^{22}\text{Na}^+$ were also performed across control and EPEC-infected monolayers using the methodology described for $^{36}\text{Cl}^-$ fluxes.

Statistical analysis. Data are presented as means $\pm$ SE. Student's *t*-test was used to compare data. Significance was defined as $P \leq 0.05$.

RESULTS

Effect of EPEC on baseline and secretagogue-stimulated I_{sc} . Confluent T84 monolayers (0.33 cm²) were infected with 2×10^7 midlog growth phase EPEC, and I_{sc} was measured at 15- to 30-min intervals for up to 6 h. At no point in time did EPEC stimulate the I_{sc} above baseline (data not shown). To determine whether EPEC infection influenced the I_{sc} response to secretagogues, uninfected and infected monolayers were challenged with either the Ca^{2+} -mediated secretagogue carbachol (10^{-4} M) or the cAMP-mediated secretagogue forskolin (10^{-6} M). As shown in Fig. 1, EPEC infection significantly decreased I_{sc} in response to both carbachol and forskolin compared with uninfected controls. Although EPEC has been previously demonstrated to increase tight junction permeability, that attenuation in I_{sc} could not be attributed to alterations in resistance was shown in two ways. First, the changes in secretagogue-induced I_{sc} could be demonstrated before the EPEC-associated decrease in resistance occurred (Fig. 2). Second, the EPEC mutant UMD864, a deletion of the *espB* gene necessary for the activation of several host

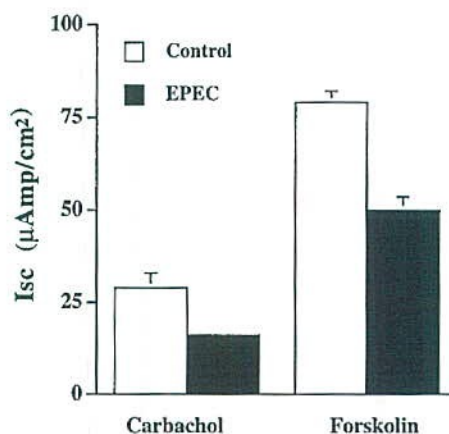


Fig. 1. Effect of enteropathogenic *Escherichia coli* (EPEC) infection on stimulated short-circuit current (I_{sc}). T84 monolayers, both uninfected controls and EPEC infected (3 h), were stimulated with either carbachol (10^{-4} M) or forskolin (10^{-6} M). Values represent means $\pm$ SE for peak I_{sc} (~ 2 min for carbachol and 10 min for forskolin; $n = 8$ –18 monolayers, $P < 0.007$ control vs. infected).

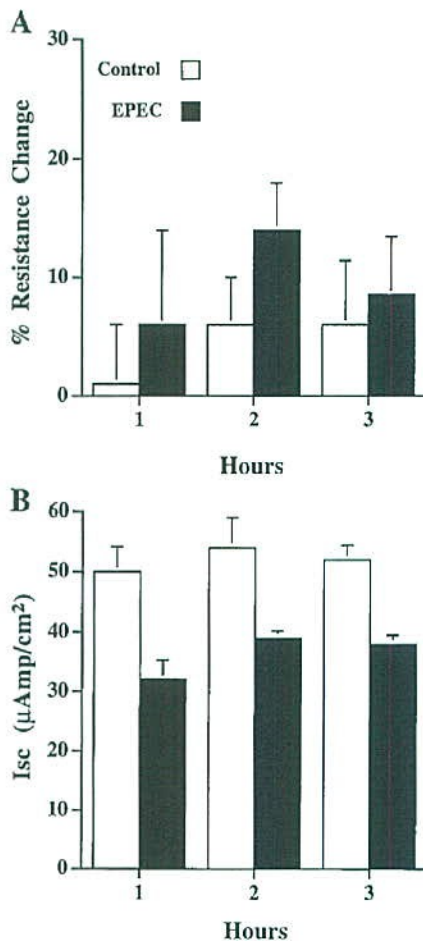


Fig. 2. Alterations in barrier function do not account for diminished I_{sc} . *A*: effect of EPEC on transepithelial electrical resistance after 1, 2, and 3 h of infection. Even after 3 h of infection with EPEC, there is no significant change in resistance. *B*: EPEC-induced diminution in forskolin-stimulated I_{sc} is not related to decreased resistance. At 1, 2, and 3 h postinfection with EPEC when resistance remains unchanged, a 25–35% decrease in I_{sc} is observed. For *A* and *B*, $n = 6$ and $P < 0.01$.

signaling pathways, does not alter transepithelial resistance ($+20 \pm 8$ vs. $-6 \pm 5\%$ change in resistance for UMD864 vs. control), yet it still causes the observed attenuation in forskolin-stimulated I_{sc} (90 ± 4 vs. $62 \pm 4 \mu\text{A}/\text{cm}^2$ for control and UMD864, respectively). Sterile bacterial supernatants had no effect on secretagogue-stimulated I_{sc} (data not shown), suggesting that a soluble factor is not responsible.

Diminished Cl^- secretion does not account for the decrease in secretagogue-induced I_{sc} . T84 monolayers have been widely used as a model cell line in which to study Cl^- secretion (1). Because EPEC has been shown to diminish the I_{sc} response in T84 monolayers challenged with secretagogues, it has been presumed that decreased Cl^- secretion is responsible. This hypothesis, however, has not been directly tested. Electrogenic Cl^- secretion requires the coordinated activity of several ion transporters. These include the apical Cl^- channel and basolateral $\text{Na}^+-\text{K}^+-\text{ATPase}$, $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter, and K^+ channels. Our approach, therefore, was

to systematically evaluate the effect of EPEC on the transport components that contribute to apical Cl^- secretion.

Cl^- channel activity was investigated first by performing ^{125}I efflux studies in uninfected and infected T84 cell monolayers. This approach has been previously validated as an acceptable method for examining apical Cl^- channel activity in T84 cells (25). Surprisingly, there was no significant difference in ^{125}I efflux from uninfected and EPEC-infected monolayers stimulated with either carbachol (0.068 ± 0.005 vs. 0.075 ± 0.014 for control and EPEC-infected monolayers, respectively) or forskolin (Fig. 3). It was suggested previously that the diminished I_{sc} response in EPEC-infected monolayers might be secondary to inhibition of the basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter, known to be regulated by the actin (19) and myosin (15) cytoskeleton. No difference in cotransporter activity, however, was detected as assessed by ^{86}Rb uptake studies in response to carbachol (9.6 ± 1.6 vs. $8.1 \pm 0.7 \text{ nmol } \text{K}^+ \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for control and EPEC-infected monolayers, respectively; $n = 6$; $P = 0.34$) or forskolin (Fig. 4A). Similarly, no diminution in basolateral K^+ channel activity in EPEC-infected monolayers was seen as determined by basolateral ^{86}Rb efflux studies (Fig. 4B). Taken together, these data demonstrate that each transport component required for apical Cl^- secretion to occur is fully functional in EPEC-infected monolayers.

To confirm that EPEC infection did not alter net Cl^- secretion, unidirectional $^{36}\text{Cl}^-$ flux studies were performed in modified Ussing chambers. These data are presented in Table 1. In control monolayers stimulated with forskolin, the I_{sc} predicted by the net secretion of Cl^- is $\sim 40\%$ less than the corresponding measured I_{sc} . In EPEC-infected monolayers, the predicted and measured I_{sc} more closely corresponded, showing that only 17% of I_{sc} is not attributable to Cl^- secretion. There was no significant difference in forskolin-stimulated, unidirectional, or net movement of $^{36}\text{Cl}^-$ across control and

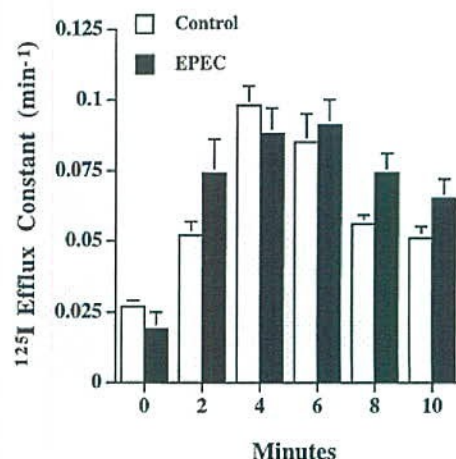


Fig. 3. EPEC infection does not alter Cl^- channel activity. Control and infected T84 cell monolayers were loaded with ^{125}I as described in METHODS. ^{125}I efflux was then determined at 2-min intervals following stimulation with forskolin ($n = 10$ –12, $P > 0.05$ for all time points).

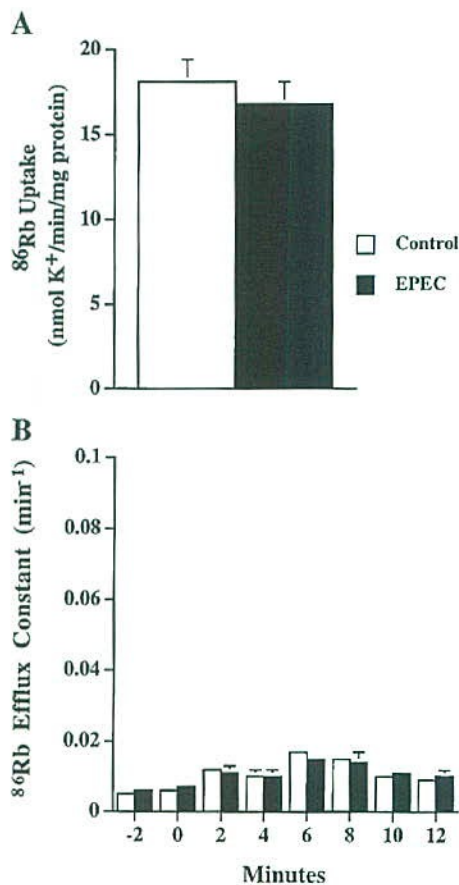


Fig. 4. EPEC infection does not alter $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (A) or basolateral K^+ channel (B) activity. A: $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter activity was assessed in control and EPEC-infected monolayers in response to forskolin stimulation. Uptakes were allowed to proceed for 3 min ($n = 8$, $P = 0.2$). B: activity of basolateral K^+ channels was determined by measuring forskolin-stimulated ^{86}Rb efflux from isotope-loaded monolayers as described in METHODS. There is a small but significant increase in ^{86}Rb efflux in response to forskolin as has been reported previously. There is, however, no difference between the responses in control and EPEC-infected monolayers ($n = 8$, $P = 0.5$).

EPEC-infected monolayers. It should be noted that, in general, I_{sc} measured in the modified Ussing chamber is typically lower than that measured in the simplified apparatus. The degree of difference in I_{sc} between control and infected monolayers, however, is consistent. These findings confirm that the attenuation in secretagogue-stimulated I_{sc} cannot be attributed to a decrease in apical Cl^- secretion. They also suggest that secretion of another anion contributes significantly to I_{sc} in secretagogue-stimulated T84 monolayers.

Neither stimulation of apical K^+ secretion nor inhibition of Na^+ absorption accounts for diminished I_{sc} in EPEC-infected monolayers. Because diminished Cl^- secretion did not explain the attenuated I_{sc} response in EPEC-infected monolayers, alternative explanations were explored. First, the possibility that apical K^+ secretion might be stimulated, thus blunting measured I_{sc} , was examined. To test this possibility, apical ^{86}Rb efflux studies were performed. No difference was seen, however, between uninfected and infected monolayers (0.001 ± 0.0001 vs. 0.001 ± 0.0003 efflux rate constants for control and EPEC-infected monolayers; $n = 8$; $P = 0.1$). Second, the role of altered Na^+ transport was explored in three ways. First, if inhibition of Na^+ absorption were the cause of blunted I_{sc} , then removal of Na^+ from the apical reservoir should increase stimulated I_{sc} back to the level seen in uninfected control monolayers. Removal of apical Na^+ had no effect on I_{sc} . Second, apical Na^+ absorption in the intestine occurs, in part, via the Na^+/H^+ exchanger 3 (NHE3). To inhibit NHE3, amiloride (100 μM) was added to the apical reservoir of monolayers before stimulation with secretagogues. The I_{sc} response in EPEC-infected monolayers was not altered by amiloride pretreatment. Amiloride also blocks Na^+ channels; therefore, taken together, these data indicate that neither inhibition of Na^+ absorption by NHE3 nor inhibition of Na^+ channels accounts for the attenuation in I_{sc} associated with EPEC infection. Finally, that altered Na^+ transport was not responsible for the observed diminution in I_{sc} was shown by performing unidirectional $^{22}\text{Na}^+$ fluxes (Table 1). There was minimal movement of Na^+ in response to cAMP-mediated secretagogues as has been demonstrated previously (5). These data also confirm the integrity of the tight junctions, dispelling the possibility that enhanced paracellular permeability might account for some of the difference in I_{sc} between control and EPEC-infected monolayers.

EPEC infection alters HCO_3^- transport. Because investigations regarding the effect of EPEC on each of the major ions transported by intestinal epithelium revealed no significant differences, a role for HCO_3^- was considered. HCO_3^- transport by the basolateral $\text{Na}^+\text{-HCO}_3^-$ cotransporter is electrogenic; therefore, the impact of removal of basolateral HCO_3^- on forskolin-induced I_{sc} was examined in control and EPEC-infected monolayers. As shown in Fig. 5, removal of basolateral HCO_3^- decreases I_{sc} in EPEC-infected monolayers to a much greater extent than it does in controls. These findings suggest that a basolateral, HCO_3^- -dependent

Table 1. Unidirectional and net Cl^- and Na^+ fluxes across control and EPEC-infected T84 monolayers stimulated with forskolin

	$J_{\text{m} \rightarrow \text{s}}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{\text{s} \rightarrow \text{m}}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{\text{net}}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	Predicted I_{sc} $\mu\text{A}/\text{cm}^2$	Measured I_{sc} $\mu\text{A}/\text{cm}^2$	R $\Omega \cdot \text{cm}^2$	$J_{\text{m} \rightarrow \text{s}}^{\text{Na}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{\text{s} \rightarrow \text{m}}^{\text{Na}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{\text{net}}^{\text{Na}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$
Control	1.17 ± 0.15	2.22 ± 0.12	-1.05 ± 0.03	27	45 ± 7.3	$1,450 \pm 159$	0.39 ± 0.16	0.42 ± 0.24	-0.03 ± 0.01
EPEC infected	0.87 ± 0.28	1.98 ± 0.2	-1.11 ± 0.08	29	35 ± 3.4	$1,305 \pm 227$	0.46 ± 0.24	0.41 ± 0.24	$+0.05 \pm 0.02$

Values are means $\pm$ SE. EPEC, enteropathogenic *E. coli*. $J_{\text{m} \rightarrow \text{s}}^{\text{Cl}}$, $J_{\text{s} \rightarrow \text{m}}^{\text{Cl}}$, and $J_{\text{net}}^{\text{Cl}}$, mucosal-to-serosal, serosal-to-mucosal, and net Cl^- flux, respectively. $J_{\text{m} \rightarrow \text{s}}^{\text{Na}}$, $J_{\text{s} \rightarrow \text{m}}^{\text{Na}}$, and $J_{\text{net}}^{\text{Na}}$, mucosal-to-serosal, serosal-to-mucosal, and net Na^+ flux, respectively. I_{sc} , short-circuit current; R , resistance.

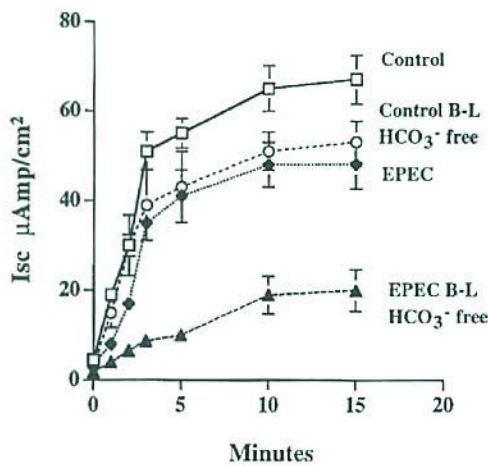


Fig. 5. Effect of removal of basolateral (B-L) HCO_3^- on forskolin-induced I_{sc} . HCO_3^- was removed from the basolateral reservoir of uninfected control and EPEC-infected T84 monolayers as described in METHODS. I_{sc} was followed to peak response in all groups. Removal of HCO_3^- from the basolateral medium decreases forskolin-stimulated I_{sc} in control monolayers by 25–30%. In fact, removal of basolateral HCO_3^- from control monolayers produces an I_{sc} response curve that is identical to that seen with EPEC infection. In contrast, HCO_3^- removal from EPEC-infected monolayers further decreases the I_{sc} response.

process contributes significantly to I_{sc} generated by EPEC-infected monolayers.

It is becoming increasingly apparent that, in several different epithelia, secretagogue-stimulated I_{sc} is a combination of Cl^- and HCO_3^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) (3, 12, 13). In fact, at least in duodenal epithelium, it appears that CFTR may function not only as a channel for HCO_3^- secretion but may also regulate the activity of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the apical membrane (3). The dogma in T84 cell monolayers has been that I_{sc} represents only Cl^- secretion. A role for HCO_3^- secretion in the T84 cell model, however, has not been explored. In view of recently published data regarding cAMP-stimulated HCO_3^- secretion via CFTR, we examined the contribution of HCO_3^- to stimulated I_{sc} in control and EPEC-infected monolayers. Initial studies were unidirectional $^{36}\text{Cl}^-$ fluxes in the total absence of HCO_3^- (Table 2). The removal of HCO_3^- decreased by ~50% net Cl^- secretion from both uninfected control and EPEC-infected monolayers. Under these circumstances, the predicted and measured I_{sc} corresponded perfectly in uninfected monolayers. In EPEC-infected monolayers, however, some discrepancy persists that suggests that the decrease in I_{sc} may not be attributable to a single event.

EPEC-induced changes in I_{sc} are not driven by mucosal acidification. In this model, the apical surface of T84 cells is infected by EPEC. Under these conditions, the medium in the apical reservoir becomes acidified over time. HCO_3^- secretion via CFTR has been demonstrated to be stimulated by mucosal acidification (13). When I_{sc} measurements are taken using our model, however, the original medium is removed and replaced with Ringer solution (pH 7.4) before challenge with secretagogues. To confirm that apical acidification is not the factor driving the altered I_{sc} response, additional experiments were performed. First, acidified culture medium from infected T84 cells was removed, filtered, and added to new monolayers for 3 h. These new monolayers were then challenged with either forskolin or carbachol. There was no significant difference in the I_{sc} response between control and supernatant-treated monolayers stimulated with forskolin (94 ± 4 vs. 86 ± 11 $\mu\text{A}/\text{cm}^2$ for control and infected monolayers; $n = 8$; $P = 0.1$) or carbachol (61 ± 4 vs. 56 ± 11 $\mu\text{A}/\text{cm}^2$ for control and EPEC-infected monolayers, respectively; $n = 8$; $P = 0.5$).

In other experiments, the mucosal medium of uninfected control monolayers was acidified to the pH seen in EPEC-infected monolayers (6.8), incubated for 3 h, and then challenged with the secretagogue. Again, no difference in the I_{sc} response was observed (data not shown). These data indicate that mucosal acidification by EPEC is not responsible for the observed difference in net ion transport.

DISCUSSION

The pathophysiology underlying EPEC-associated diarrhea appears to be much more complex than initially realized. The originally described morphological lesion, which included microvillus degeneration, seemed initially to provide a logical explanation for the resultant diarrhea. That is, it was presumed that the loss of crucial microvillar brush-border enzymes resulted in malabsorption and osmotic diarrhea. Subsequent data resulting from several different avenues of investigation, however, suggest that this is not the case. For example, human volunteer studies have shown that the onset of diarrhea following the ingestion of EPEC occurs as early as 4 h (6), too short a time for the sequence of attachment, effacement, and malabsorption to occur. Also, EPEC adheres as microcolonies, not individual bacteria, thus infecting only a portion (~50%) of enterocytes (14). As such, a substantial number of unaffected absorptive cells remain. Furthermore, only those microvilli in close proximity to adherent EPEC are altered, leaving some normal-appearing microvilli

Table 2. Forskolin-stimulated unidirectional and net Cl^- fluxes in control and EPEC-infected T84 monolayers in the absence of HCO_3^-

	$J_{m \rightarrow a}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{a \rightarrow m}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	$J_{\text{net}}^{\text{Cl}}$ $\mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$	Predicted I_{sc} , $\mu\text{A}/\text{cm}^2$	Measured I_{sc} , $\mu\text{A}/\text{cm}^2$
Control	0.61 ± 0.12	1.18 ± 0.2	-0.57 ± 0.07	15	15.5 ± 0.85
EPEC infected	0.59 ± 0.16	1.15 ± 0.24	-0.56 ± 0.05	15	11 ± 1.18

Values are means $\pm$ SE.

on the infected enterocyte. Last, although EPEC infection undoubtedly accelerates the normal process of vesicular shedding of microvillar digestive enzymes (2), there is evidence to suggest that the enterocyte may respond by a compensatory increase in the synthesis of such enzymes (7). These data, in addition to more recent studies that examine in detail the impact of EPEC infection on intestinal epithelial cell function, suggest that the pathophysiology of EPEC-associated diarrhea is quite complex and cannot be simply attributed to loss of brush-border enzymes.

The elucidation of the pathophysiology underlying EPEC-associated diarrhea is further complicated by the finding that EPEC does not induce active ion secretion. In an early study by Moon et al. (20), it was reported that infection of ligated ileal loops by EPEC in both pigs and rabbits did not induce fluid accumulation despite the presence of the attaching and effacing lesion. Interestingly, infection was found more frequently and was characterized as being more extensive in the large vs. the small intestine. Colonic loop experiments were not performed in this study.

More recently, attention has shifted to examining the effects of EPEC specifically on its host target cell, the intestinal epithelial cell. These studies have yielded interesting information. In contrast to the usual paradigm of bacteria-induced Cl^- secretion, EPEC has been shown herein and by others (23) to not activate this process. In fact, net ion secretion stimulated by classic secretagogues is blunted in EPEC-infected monolayers. The results presented here suggest that such attenuation is a global phenomenon in that both Ca^{2+} - and cAMP-stimulated I_{sc} values are decreased. Philpott et al. (23), however, demonstrated attenuated I_{sc} only when stimulated with forskolin, not carbachol. This scenario is reminiscent of what occurs when the actin cytoskeleton is stabilized with agents such as phalloidin (19). In this case, diminished I_{sc} in response to forskolin is attributable to inhibition of the basolateral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. It was, therefore, hypothesized that the effect of EPEC on forskolin-stimulated I_{sc} , which was presumed to reflect diminished Cl^- secretion, may be linked to this same mechanism. These investigators, however, did not examine the effect of EPEC on Cl^- secretion specifically but only examined I_{sc} , a measurement of net ion transport. Furthermore, EPEC has not been shown to alter basal actin, a component demonstrated to regulate $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter activity.

The data presented here show that the diminution in stimulated I_{sc} caused by infection with EPEC is, surprisingly, not due to diminished Cl^- secretion. This was demonstrated by showing that each specific transporter participating in apical Cl^- secretion, including apical Cl^- channels and the basolateral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, was not altered by EPEC. In addition, Cl^- flux studies performed in Ussing chambers confirmed that net Cl^- secretion was identical in control and infected monolayers. It should be noted, however, that a recent publication reported that EPEC infection of Caco-2 monolayers induced a rapid but transient

increase in I_{sc} , a portion (~25%) of which was Cl^- dependent (4). The peak change in I_{sc} was seen at 10-min postinfection; however, a model of synchronized infection was employed whereby bacteria were centrifuged onto the cell surface. Obviously, the differences in the model employed, both cell line and method of infection, could account for the variability in results.

Our studies also suggest that altered Na^+ absorption does not account for the diminished I_{sc} seen in EPEC-infected monolayers. Neither removal of Na^+ from the apical buffer nor treatment with amiloride, which inhibits NHE3 as well as Na^+ channels, altered the attenuated I_{sc} response. In addition, Na^+ flux studies showed no difference in Na^+ transport.

It is becoming increasingly apparent that CFTR serves not only as a conductor for the active secretion of Cl^- but also for HCO_3^- (3, 12, 13). The role of CFTR in HCO_3^- secretion is, however, more complex. Much like Cl^- secretion, the secretion of HCO_3^- requires the orchestration of several transporters, including an anion channel, apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger, and basolateral uptake by the $\text{Na}^+\text{-HCO}_3^-$ cotransporter. Intracellular HCO_3^- may also be generated through the action of carbonic anhydrase. The effect of cAMP and Ca^{2+} stimulation on these various components involved in HCO_3^- secretion is just beginning to be investigated.

The contribution of HCO_3^- to secretagogue-stimulated I_{sc} in T84 cells has not been addressed. Our studies suggest, contrary to popular dogma, that HCO_3^- may be responsible for up to 40% of forskolin-stimulated I_{sc} , the difference between calculated and measured I_{sc} shown herein. In reviewing the data published as a part of the initial characterization of the transport properties of T84 cells (5), the measured I_{sc} was 20% greater than that predicted from the net secretion of Cl^- . This unexplained I_{sc} may be attributable to HCO_3^- secretion. Our Cl^- flux studies performed in the absence of HCO_3^- showed that both predicted and measured I_{sc} dropped significantly. This does not necessarily imply that HCO_3^- secretion directly accounts for nearly one-half of the generated I_{sc} . Instead, it has been proposed that the activities of CFTR and the apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger are linked (3). One offered explanation of this interaction is that the $\text{Cl}^-/\text{HCO}_3^-$ exchanger serves to recycle Cl^- through the apical membrane (3). If $\text{Cl}^-/\text{HCO}_3^-$ activity is interrupted, either by direct inhibition or removal of HCO_3^- , then there may be a decrease in net Cl^- secretion as we observed in our flux studies. Under these conditions, measured and predicted I_{sc} corresponded perfectly.

Regarding the effect of EPEC infection on stimulated net ion transport, our studies clearly demonstrate that diminished Cl^- secretion does not account for the observed difference in I_{sc} . Examination of the influence of EPEC on the transport of other major ions, namely Na^+ and K^+ , failed to reveal any change. That diminished transepithelial resistance was not responsible for the measured changes in active ion transport was shown in two ways. First, alterations in secretagogue-stimulated I_{sc} were demonstrated before changes in

permeability, as shown in Fig. 2. Second, if altered paracellular permeability were responsible, the flux rate of Na^+ would have increased. Furthermore, a mutant strain of EPEC known to not decrease epithelial resistance still induces alterations in I_{sc} .

How, then, might the EPEC-induced diminution in I_{sc} be explained? In that the degree of decrease in net ion transport is quite substantial and alterations in the transport of none of the major ions, including Cl^- , Na^+ , and K^+ , appear to account for this change, HCO_3^- is a likely candidate. When HCO_3^- is removed from uninfected control monolayers, secretagogue-stimulated I_{sc} decreases significantly, ranging from 20 to 40%. In this case, I_{sc} approximates that seen in EPEC-infected monolayers. We, therefore, propose that EPEC is altering the transport of HCO_3^- . Whether this change is being effected at the apical or basolateral membrane cannot be distinguished at this point. Also, the complex interaction that appears to exist between CFTR and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger makes the regulation of HCO_3^- secretion difficult to unravel. Clarke and Harline (3) suggest that, in the duodenal epithelia, the relative contribution of CFTR as a conductor of HCO_3^- and as a "facilitator" of apical $\text{Cl}^-/\text{HCO}_3^-$ exchange likely changes in response to cues from the luminal environment. In addition, the activities of the basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter and intracellular carbonic anhydrase must be considered. To reconcile the specific mechanisms by which EPEC interferes with HCO_3^- transport, many additional studies will be required. EPEC-infected intestinal epithelial cells should, however, provide an interesting model for deciphering the complex pathways that regulate HCO_3^- secretion.

Although these studies enhance our understanding of the effects of EPEC infection on active ion transport, they do not fit the usual paradigm for diarrhea that occurs in response to infection by enteric pathogens. It has been shown that with time EPEC increases the permeability of tight junctions by inducing the phosphorylation of myosin light chain (27). This event may contribute to the production of diarrhea, but it is likely that other factors are involved as well.

In vivo, EPEC infection induces the recruitment of inflammatory cells, primarily neutrophils, not only into the lamina propria but also across intact crypts (20). Activated neutrophils produce 5'-AMP, which is converted by epithelial cell 5'-ectonucleotidase to adenosine, a potent secretagogue (17). In our in vitro model, EPEC infection also attenuates I_{sc} in response to adenosine (unpublished observation). In vivo, however, EPEC attaches primarily to surface epithelial cells and less often to crypt cells, causing severe villous atrophy and crypt hyperplasia (22). Neutrophils tend to accumulate in intestinal crypts where it would be possible for uninfected crypt cells in the in vivo situation to demonstrate a full secretory response to neutrophil-derived adenosine.

In summary, the studies presented herein confirm that EPEC infection of intestinal epithelial cells diminishes net ion transport, as measured by I_{sc} in response to classic secretagogues. Unexpectedly, our results

clearly show that this decrease in net ion transport does not represent diminished Cl^- secretion as one might predict. Instead, it appears that EPEC alters HCO_3^- secretion and possibly perturbs other HCO_3^- -dependent transport processes. Additional studies are needed to further define these transport changes.

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Enteropathogenic *Escherichia coli* EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier

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Summary

EspG, a secreted effector of enteropathogenic *Escherichia coli* (EPEC), as well as its homologue Orf3, has been shown to disrupt microtubules (MTs) in fibroblasts and non-polarized epithelial cells. The roles of MTs and the effects of MT disruption in these cell types differ significantly. The aim of this study was to investigate the effects of EspG on polarized, host target intestinal epithelial cells. Immunofluorescent labelling of tubulin showed that EPEC caused progressive fragmentation and loss of the MT network in cells harbouring attached organisms. Immunoblots of proteins extracted from EPEC-infected cells showed a corresponding loss of α -tubulin. Type III secretion system (TTSS)-deficient strains had no effect on MT suggesting TTSS dependence. Mutation of *espG*, but not *espF* or *map*, ablated EPEC's effects on MTs for up to 6 h. Ectopic expression of EspG in HeLa cells caused MT disruption. While deletion of *espG* alone had no effect on the EPEC-induced decrease in transepithelial electrical resistance (TER), mutation of both *espG* and *orf3* significantly delayed the kinetics of this response. Complementation of the double mutant with *espG* alone restored the kinetics of TER drop to that of wild type. Herein, we describe a previously unrecognized phenotype for the EPEC effectors EspG and Orf3.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a diarrhoeic pathogen that attaches to the gastrointestinal mucosa and secretes several proteins into host intestinal epithelial cells (IEC) through a type III secretion system (TTSS) (McDaniel *et al.*, 1995; Celli *et al.*, 2000). These effector molecules cause a number of pathophysiological changes in IECs including the recruitment and rearrangement of actin and other cytoskeletal proteins, to form attaching and effacing (A/E) lesions (Celli *et al.*, 2000; Gruenheid and Finlay, 2003), loss of the epithelial tight junction (TJ) barrier (Spitz *et al.*, 1995; Hecht, 2001; Muza-Moons *et al.*, 2003) and inflammation (Savkovic *et al.*, 1997; 2001; 2003). Currently several secreted effector molecules have been identified: Tir, Map, EspF, EspG, EspH, EspI (NleA), NleB-G and Cif (Elliott *et al.*, 2001; Kenny, 2002; Tu *et al.*, 2003; Deng *et al.*, 2004; Mundy *et al.*, 2004). These effectors, except for EspI (NleA), NleB-G and Cif, along with the TTSS are encoded on a pathogenicity island called the locus of enterocyte effacement (LEE) (Clarke *et al.*, 2003). The LEE genes are sufficient to confer pathogenicity on a non-pathogenic strain of *E. coli*; however, other genes that lie outside the LEE might also be involved in infection (McDaniel and Kaper, 1997).

EspG has been identified as a secreted protein. Clues to the function of EspG were found in its homology (21% identity, 40% similarity) to *Shigella flexneri* VirA (Elliott *et al.*, 2001). VirA promoted *Shigella* invasion by binding tubulin and stimulating microtubule (MT) destruction (Uchiya *et al.*, 1995; Yoshida *et al.*, 2002; Yoshida and Sasakawa, 2003). MT destabilization promoted rapid localized membrane ruffling that was believed to promote bacterial internalization. A *virA* mutant had one-fifth the survival rate in invasion assays as wild-type *Shigella*. EPEC *espG* complemented a *S. flexneri virA* mutant and restored the intracellular persistence phenotype (Elliott *et al.*, 2001). Although EPEC is minimally internalized into non-intestinal cell lines *in vitro*, unlike *Shigella*, it is not a true invasive pathogen *in vivo* (Donnenberg *et al.*, 1989; Donnenberg *et al.*, 1990; Jepson *et al.*, 2003). A bacterial persistence assay with wild-type EPEC and Δ *espG* showed no significant differences between the two strains

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(Elliott *et al.*, 2001). EPEC also encodes Orf3, an EspG homologue (43% identity, 63% similarity) on the EspC pathogenicity islet. EPEC *orf3* also complemented *virA* deletion in *S. flexneri* suggesting functional similarity to EspG (Elliott *et al.*, 2001).

Recently, Matsuzawa *et al.* (2004) reported that EPEC EspG and Orf3 cause focal disruption of MTs in Swiss 3T3 fibroblasts and non-polarized HeLa cells immediately beneath attached microcolonies. They further demonstrated that disruption of MTs caused release of guanine nucleotide exchange factor (GEF)-H1, activation of RhoA and an increase in actin stress fibres in these non-polarized cells. However, a recent comparison of the cytoskeletal association of GEF-H1 in different cell types revealed the lack of association of endogenous GEF-H1 with MTs in non-transfected polarized epithelial lines including MDCK and Caco-2, non-polarized epithelial cells, HeLa and non-epithelial cells 3T3 (Benais-Pont *et al.*, 2003). Instead, endogenous GEF-H1 was found to localize to different structures in these cells; TJs in polarized epithelial cells and to stress fibres in fibroblasts and non-polarized epithelial cells. Therefore, the aim of our study was to examine the effects of EspG, and its homologue Orf3, on polarized IECs and determine its role in EPEC pathogenesis.

Results

EPEC, but not Δ espG, disrupts MT in IECs

In non-dividing polarized epithelial cells, most MTs are localized in a dense web-like network directly under the apical membrane with some strands running parallel to the lateral membrane (Nelson and Yeaman, 2001). In order to determine the effect of wild-type EPEC infection on the MT network of polarized human IECs, T84 monolayers were infected for up to 5 h. As shown in Fig. 1A, MTs progressively disappeared from an increasing number of cells after infection with EPEC; corresponding phase-contrast images showed that the monolayers are intact. The same effect was seen in HeLa and Caco-2 cell lines (data not shown). Lesser multiplicities of infection

(moi) delayed, but did not prevent, the MT phenotype. The EPEC-induced MT loss was more dramatic than the limited effects described for *Shigella* or for EPEC infection of fibroblasts where disruption was focal, seen only in areas immediately surrounding the internalized or attached bacteria (Yoshida *et al.*, 2002; Matsuzawa *et al.*, 2004). In striking contrast to wild-type EPEC, the Δ espG strain had little to no effect on MTs even at 6 h after infection (Fig. 1B). These results indicate that EspG plays an important role in MT disruption in IECs. Complementation of *espG* (Δ espG/pCVD453) restored the MT phenotype as shown in Fig. 1C. Longer infection caused further disruption similar to a wild-type infection (data not shown). A non-pathogenic *E. coli* strain, HS-4, did not affect MTs (data not shown).

Previous studies have shown that *orf3* or *espG* can complement a *Shigella virA* mutant (Elliott *et al.*, 2001; Matsuzawa *et al.*, 2004). In order to determine whether *Shigella virA* could complement EPEC Δ espG, *S. flexneri* 2a *virA* was cloned and transformed into the Δ espG mutant strain. Monolayers were infected with the resulting strain, GH292, and immunofluorescent staining for α -tubulin was performed and compared with control and Δ espG-infected cells. As seen in Fig. 1D, *virA* complemented EPEC-induced MT disruption, indicating that VirA can substitute for EspG in EPEC. Curiously, in contrast to the VirA-dependent focal lesions in MT induced by *Shigella*, the VirA-complemented Δ espG strains mediated generalized MT loss, similar to that caused by EPEC (Fig. 1A).

Depolymerization of MTs precedes degradation and is associated with A/E lesions

Microtubules are dynamic structures existing in a state of constant elongation and disassembly, termed 'dynamic instability' (Valiron *et al.*, 2001). The loss of MTs in infected monolayers could be either due to decreased elongation, increased disassembly, sometimes called 'catastrophe' (Valiron *et al.*, 2001), or degradation of tubulin. Nocodazole, a MT destabilizer, causes fragmentation and depolymerization of MTs without corresponding tubu-

Fig. 1. EPEC disrupts MTs in a time-dependent manner.

A. T₈₄ cells were infected with EPEC for up to 5 h then MTs were visualized using an α -tubulin primary antibody and an Alexa Fluor 568-conjugated secondary antibody. Actin was stained using phalloidin-conjugated FITC. Representative images of control monolayers and those infected for 3 and 5 h show the progressive loss of MTs. Corresponding phase-contrast images show a healthy, intact monolayer. Actin stains of the same field show A/E lesion formation in infected monolayers. The insets highlight individual pedestals.

B. Δ espG-infected T₈₄ cells were examined over a 6 h time-course but no loss in α -tubulin staining was seen. A corresponding phase-contrast image is also shown. Actin staining again demonstrates attached microcolonies with an inset of individual pedestals.

C. Complementation of the *espG* mutant by adding the cloned *espG* gene on a multicopy plasmid (pCVD453) restored the loss of α -tubulin to levels seen with wild-type EPEC. A representative image of T₈₄ cells at 4 h shows MT disruption while the corresponding phase-contrast image shows an intact monolayer. Actin staining shows individual EPEC pedestals.

D. There is no discernible difference between the MT network of uninfected control and Δ espG-infected monolayers. Complementation of EPEC *espG* with the *Shigella* homologue *virA* restored the phenotype of MT disruption. The inset shows fragmented MTs.

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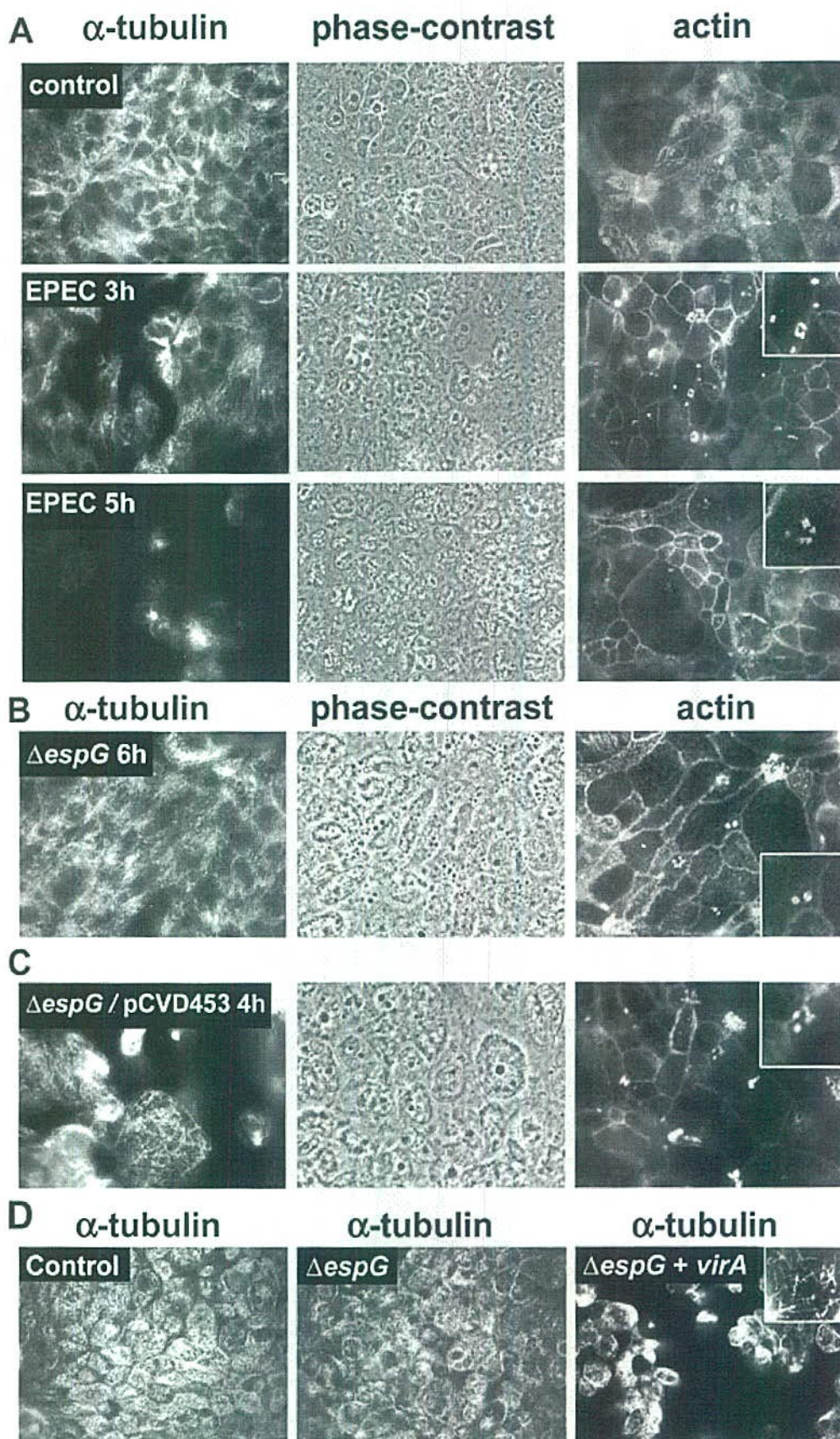




Fig. 2. EPEC degrades α -tubulin over time.

A. Proteins from EPEC-infected T₈₄ cells were extracted at hourly intervals for 5 h. α -tubulin, visualized as a 55 kDa band, was degraded over the time-course of infection. Corresponding densitometric values are provided below each lane. Actin, 42 kDa, was used as an internal control for protein loading.

B. Infection with $\Delta espG$ showed no degradation of α -tubulin for up to 6 h. Actin, 42 kDa, was used as a loading control. MW, molecular weight standards.

lin degradation (Eilers *et al.*, 1989). The total lack of tubulin staining in EPEC-infected cells suggested that tubulin subunits were being degraded and not merely disassembled. To assess whether EPEC infection caused the degradation of tubulin, T84 protein extracts from cells infected with EPEC for various times were examined by Western blotting. Figure 2A demonstrates that α -tubulin progressively decreased over 5 h of EPEC infection, corresponding to the observed morphological changes. The $\Delta espG$ strain failed to induce MT disruption for up to 6 h (Fig. 2B).

Microtubule depolymerization would be expected to precede tubulin degradation. To explore the process of EPEC-induced MT disruption in more detail, EPEC-infected T₈₄ monolayers were examined at an early time point. At 2 h after infection, a time when tubulin degradation is not obvious (Fig. 2A), MT disruption was subtle, consisting of broken MT fragments underneath A/E lesions (Fig. 3A). Although not every A/E lesion was associated with MT disruptions, it should be noted that A/E lesions do not form in a synchronized fashion; perhaps only the more mature A/E lesions are associated with disrupted MTs. Detail of the EPEC-induced fragmentation of the MT network can be clearly seen as early as 2 h after infection (Fig. 3B).

Effective TTSS is required for MT disruption by EPEC

In order to assess the role of EPEC TTSS and other specific effector molecules in MT disruption, Caco-2 cells were infected with a panel of EPEC mutants for 2 h and stained for α -tubulin (Fig. 4). In contrast to the marked effects of wild-type EPEC, TTSS mutants, including $\Delta escN$, $\Delta espA$ and $\Delta espD$, caused no MT destruction (Fig. 4A). In contrast to $\Delta escN$, $\Delta espA$ and $\Delta espD$ can secrete EspG and other proteins into the medium, although all three strains are deficient for translocation of these proteins into host cells. The absence of a phenotype for $\Delta espA$ and $\Delta espD$ therefore suggests that EspG needs to be delivered into host cells to mediate its effect. Other EPEC secreted effector mutants, $\Delta espF$ and Δmap , retained the ability to disrupt MTs (Fig. 4A). The TTSS mutant $\Delta espB$ had an attenuated phenotype causing some MT disruptions but less extensive than those induced by wild-type EPEC. $\Delta espB$ retains the ability to form pores and thus may not completely block TTSS (Shaw *et al.*, 2001). The A/E lesion-deficient mutants, Δtir and $\Delta eaeA$, also had no effect on MTs after 2 h (Fig. 4B). The absence of phenotype in these cases may result from inefficient delivery of EspG into host cells due to lack of intimate attachment, similar to what is seen with EspB (Wolff *et al.*, 1998).

The enterohaemorrhagic *E. coli* (EHEC) EspG homologue displays 98% identity with EPEC EspG. In order to determine whether EHEC also triggered MT disruption, α -tubulin immunofluorescent staining was performed on EHEC-infected monolayers. Although EHEC, like EPEC, induced MT disruption, there was a delay in appearance of this phenotype. At 3 h of EHEC infection, there was no discernible alteration in MTs (Fig. 5A), while with EPEC, MT disruption was evident in a substantial number of the cells at this time (Fig. 1A). Only after 5 h of infection with EHEC was a significant loss of MTs seen (Fig. 5A). Western blot analysis of α -tubulin from protein extracts of EHEC-infected cells confirmed this delayed phenotype

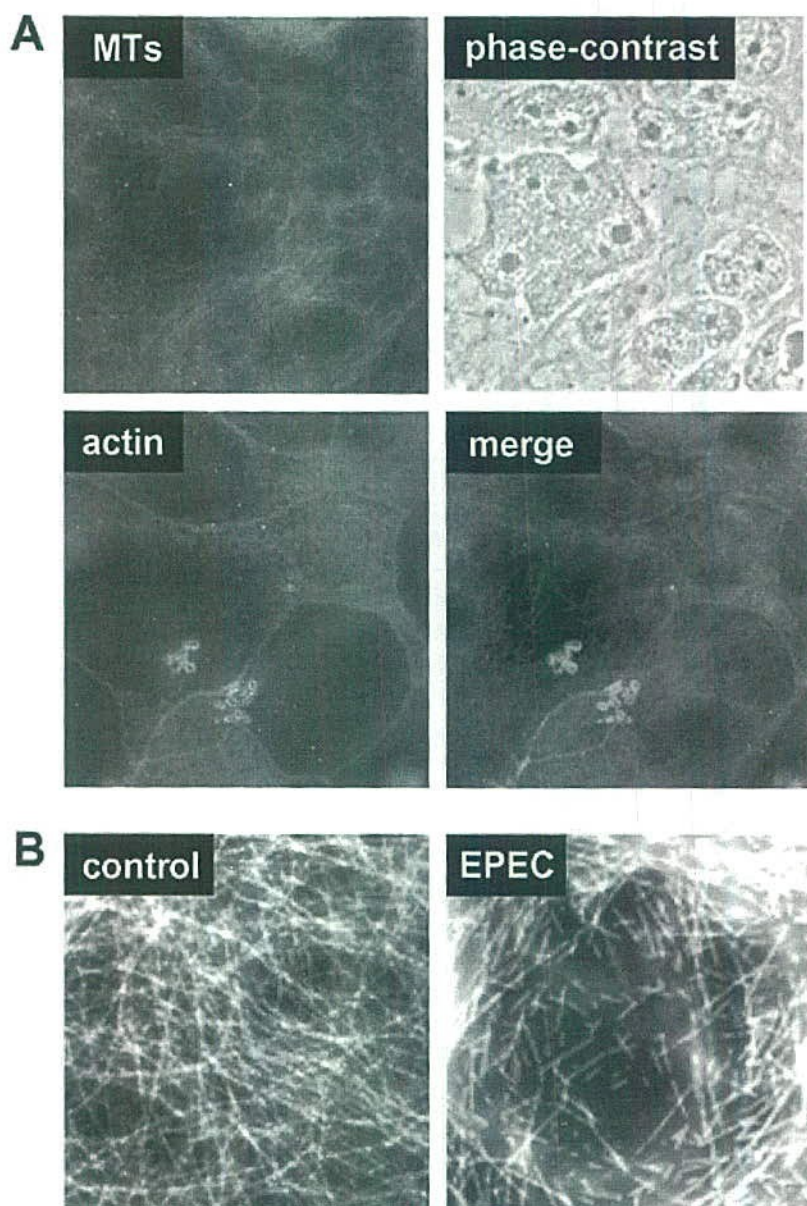


Fig. 3. EPEC infection causes fragmentation of MTs under A/E lesions.

A. Immunofluorescent staining was performed for MTs, using an α -tubulin primary antibody, or actin, using phalloidin-conjugated FITC. The first appearance of holes in MT staining was seen at 2 h after EPEC infection in T84 cells and was associated with actin pedestals. The phase-contrast image confirms the integrity of the monolayer. The combined image of actin and α -tubulin is shown in the last panel (merge).

B. High magnification of a single uninfected Caco-2 cell stained for α -tubulin showed intact MT fibres in a web-like network. Early infection with EPEC, however, induces fragmentation and depolymerization of MTs in a focal area of an individual cell.

(Fig. 5B). The delayed kinetics of α -tubulin degradation by EHEC as compared with EPEC (Fig. 2) may reflect general differences in attachment and the time-course of infection by these two pathogens (Viswanathan *et al.*, 2004).

EPEC-induced MT disruption is reversible

To confirm the integrity of the monolayer, and the presence of viable cells in the MT-disrupted regions, we assessed the reversibility of MT loss by examining the recovery of EPEC-infected monolayers after gentamicin treatment. Monolayers were infected with EPEC for 2 h,

treated with gentamicin for 1 h, and then allowed to recover for 4 h before fixation and immunofluorescent staining. As seen in Fig. 6, eradication of EPEC by treatment with gentamicin resulted in the restoration of the MT network. Recovery suggests that MT disruption is reversible and that the cells are not apoptotic or necrotic. In order to determine whether recovery of the MT network required *de novo* protein synthesis, monolayers were pre-treated with the protein synthesis inhibitor cycloheximide ($25 \mu\text{g ml}^{-1}$) for 1 h before EPEC infection and during the recovery phase. As demonstrated in Fig. 6, cycloheximide prevented recovery of the MT network indicating *de novo* protein synthesis is required.

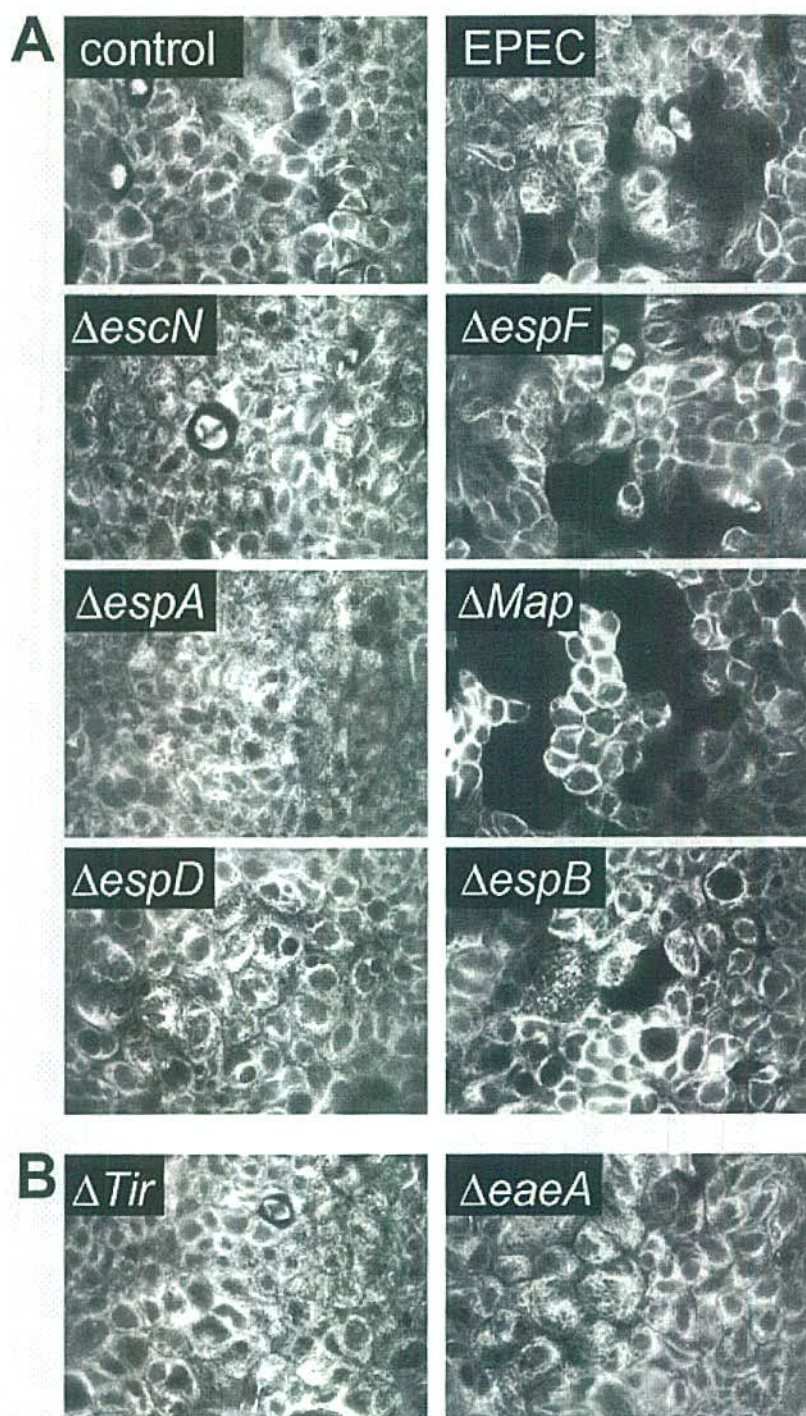


Fig. 4. Loss of MTs requires a functional TTSS and intimate attachment. Caco-2 cells were infected with various EPEC mutants for 2 h and then immunostained for α -tubulin.

A. MT loss associated with infection by ΔespF and Δmap was similar to that seen with wild-type EPEC while infection with ΔespB yielded an attenuated phenotype. Infection with ΔescN , ΔespA and ΔespD had no effect on MTs, which appeared similar to controls.

B. The A/E lesion-deficient mutants Δtir and ΔeaeA (intimin) also exhibited control phenotypes with no MT loss at 2 h.

Stabilization of MTs with paclitaxel protects against EPEC-induced MT disruption

Several pharmacological agents are used to influence MT dynamics. Paclitaxel (taxol) stabilizes MT by preventing depolymerization and arrests cellular division by inhibiting MT function (Sheppard *et al.*, 1999). Paclitaxel was

reported to be protective against *Shigella* VirA-induced MT disruption and prevented VirA from binding tubulin dimers (Yoshida *et al.*, 2002; Yoshida and Sasakawa, 2003). Immunofluorescent staining of MTs in uninfected cells treated with 100 μM paclitaxel show control-like phenotype except for abnormal mitotic spindle formation, which was expected (Fig. 7). Pretreatment of monolayers

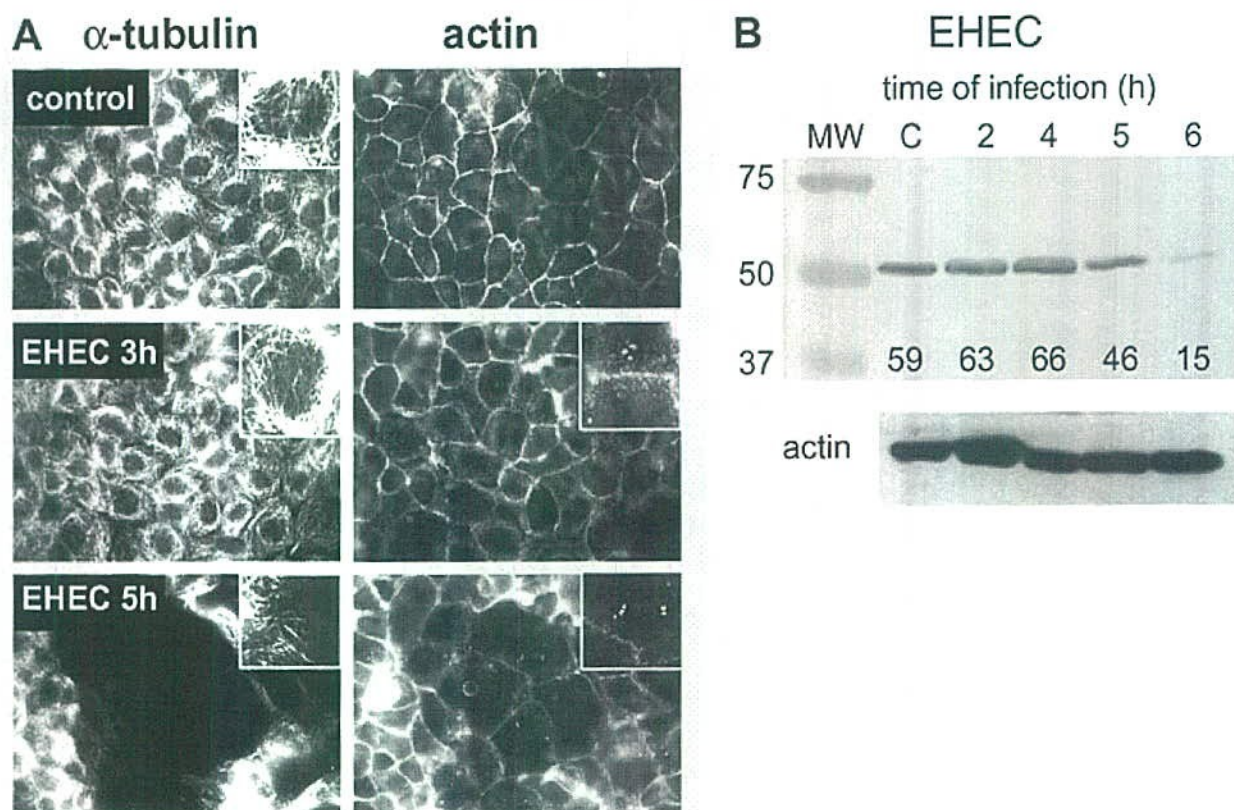


Fig. 5. EHEC infection also disrupts MTs.

A. MT disruption induced by EHEC infection occurred with a slower time-course than that caused by EPEC. At 3 h after infection of Caco-2 cells there was no effect on MTs; however, by 5 h significant loss was seen. Corresponding actin staining of the same field reveals an intact monolayer and the presence of EHEC actin pedestals (insets).

B. Western blotting for α -tubulin, visualized as a 55 kDa band, from proteins extracted from EHEC-infected T₈₄ cells shows that degradation is not detected until 5 h after infection, correlating with the α -tubulin immunostaining in (A). Actin, 42 kDa, was used as a loading control.

for 1 h with 100 μ M paclitaxel protected against EPEC-associated MT disruption at 3 h (Fig. 7).

Expression of EspG in HeLa cells is sufficient to promote MT disruption

In order to confirm that EspG alone could disrupt MTs in epithelial cells, stable inducible *espG*-transfected HeLa cells were created. A bidirectional expression plasmid allowed *espG* and a reporter gene, enhanced green fluorescent protein (EGFP), to be expressed concurrently upon induction with doxycycline. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that doxycycline-induced *espG* expression was maximal at 3 days (Fig. 8). Vector-only control cells display a healthy MT network throughout the cells (Fig. 9A). After 3 days of doxycycline induction, depolymerization and degradation of MT were seen in *espG* transfected HeLa cells, evident by EGFP expression (Fig. 9B and C), but not in non-EGFP expressing cells in the same field (Fig. 9B). Close examination of an EspG-expressing HeLa

cell shows fragmented MTs and MT loss (Fig. 9C). These results imply that EspG is sufficient to induce the EPEC-associated MT phenotype described and does not require other EPEC effectors.

Comparison of effects of $\Delta espG$ and $\Delta espG/\Delta orf3$ on intestinal epithelial phenotypes

The degree of identity between EspG and Orf3 is of particular interest. RT-PCR analyses showed that both *espG* and *orf3* expression is induced upon growth in tissue culture medium, a characteristic feature of EPEC virulence genes (data not shown). As shown in Fig. 10A, there were no discernible differences in the MT network of control monolayers or cells infected with $\Delta espG$ or the $\Delta espG/\Delta orf3$ double mutant. As disruption of MT architecture probably impacts cellular function, we also examined some hallmark functional phenotypes of infected cells using the $\Delta espG$ and $\Delta espG/\Delta orf3$ strains. EPEC-infected epithelial cells have three well-described phenotypes: A/E lesion formation (Moon *et al.*, 1983), TJ disruption

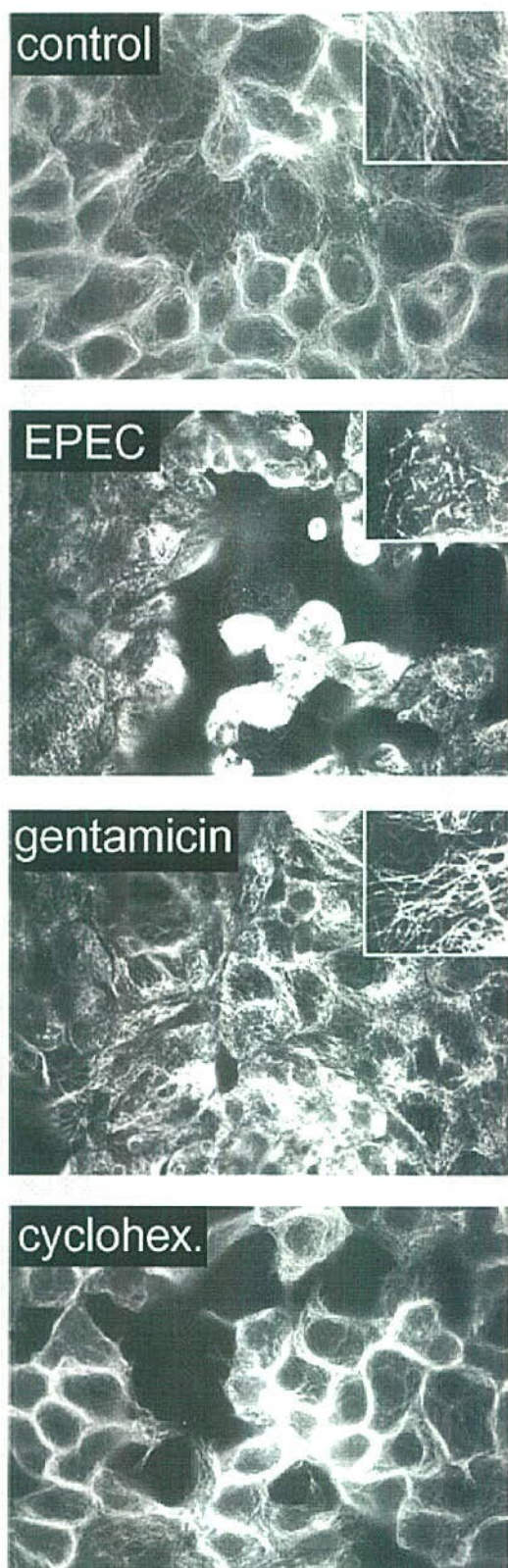


Fig. 6. EPEC-induced MT damage is reversible. Eradication of EPEC infection of Caco-2 monolayers with gentamicin restored the MT phenotype. Monolayers were infected for 2 h with EPEC (EPEC) and then treated with gentamicin (gentamicin); gentamicin alone served as the control (control). The MT network was restored as early as 4 h after eradication of EPEC. Monolayers were treated with $25 \mu\text{g ml}^{-1}$ cyclohexamide for 1 h before infection with EPEC for 2 h and during the 4 h recovery phase that followed eradication of EPEC with gentamicin (cyclohex.). The insets are magnifications of either normal intact MTs (control, gentamicin) or disrupted MTs (EPEC).

(Simonovic *et al.*, 2000; Muza-Moons *et al.*, 2003) and inflammatory responses (Savkovic *et al.*, 2001; 2003). Immunofluorescent staining for actin using FITC-conjugated phalloidin was performed after infection of cells for 2 h with wild-type EPEC, ΔespG or $\Delta\text{espG}/\Delta\text{orf3}$ (Fig. 10B). A/E lesion formation was induced by all three strains indicating that neither EspG nor Orf3 were involved in this morphological alteration. Attachment assays were also performed for each of the mutants and wild-type EPEC. There was no significant difference in the degree of attachment to cultured intestinal epithelial Caco-2 cells (data not shown).

EPEC has been shown to stimulate the inflammatory cascade in several human IEC lines (Savkovic *et al.*, 1997). EPEC activates NF- κB resulting in the transcription and secretion of the inflammatory chemoattractant interleukin (IL)-8. Infection of intestinal epithelial monolayers with wild-type EPEC, ΔespG or $\Delta\text{espG}/\Delta\text{orf3}$ increased IL-8 production to essentially the same level, although that induced by ΔespG was slightly less as compared with wild-type EPEC or the $\Delta\text{espG}/\Delta\text{orf3}$ double mutant (Fig. 10C). While the difference is statistically significant, this slightly attenuated effect is unlikely to be physiologically relevant. Dose-response studies using three different moi did not significantly change the ratios of IL-8 between wild type and the mutants (data not shown).

Transepithelial resistance (TER) of epithelial monolayers is a measure of TJ barrier function. EPEC was recently reported to activate GEF-H1 and, in turn, RhoA leading to increased formation of actin stress fibres (Matsuzawa *et al.*, 2004). This is particularly interesting in that RhoA has been shown by several investigators to play a part in the regulation of TJs (Jou *et al.*, 1998; Hopkins *et al.*, 2000). To determine whether EspG/Orf3 play a role in EPEC-induced alteration of TJ barrier function, T84 or Caco-2 monolayers were infected with wild-type EPEC, ΔespG or $\Delta\text{espG}/\Delta\text{orf3}$ and TER was monitored for up to 6 h (Fig. 11). Interestingly, while the deletion of *espG* alone had no effect on the decrease in TER by EPEC, deletion of both *espG* and *orf3* significantly delayed TJ disruption of T₈₄ monolayers as measured by TER (Fig. 11A). A similar delay in the TER phenotype by the $\Delta\text{espG}/\Delta\text{orf3}$ double mutant was seen in Caco-2 monolayers. This delay was prevented by complementation with

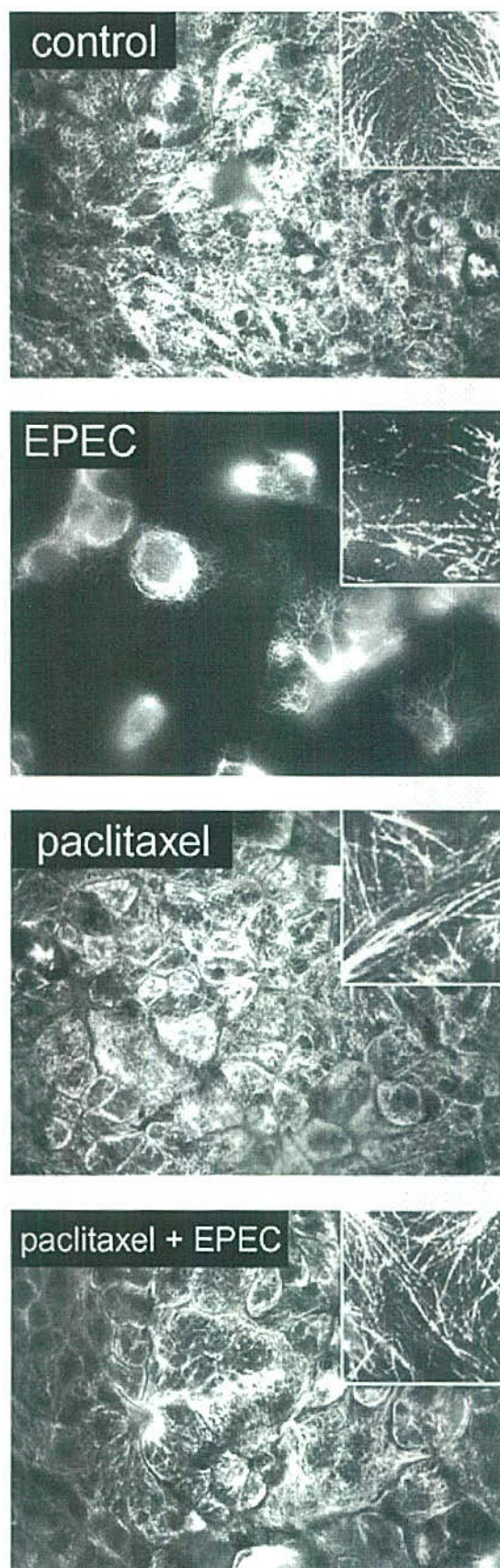


Fig. 7. Stabilization of MTs with paclitaxel prevented EPEC-induced damage. Caco-2 monolayers were pretreated with media or with 100 μ M paclitaxel, a MT stabilizing agent. Paclitaxel prevented EPEC-induced MT destabilization after 3 h of infection. The insets are magnifications of either normal intact MTs (control, paclitaxel, paclitaxel + EPEC) or disrupted MTs (EPEC).

espG alone (Fig. 11B). At 6 h after infection with the double mutant, the drop in TER approached that of wild type but was significantly different in both T84 and Caco-2 monolayers.

Discussion

In this study, we have identified a phenotype for EPEC EspG in IECs, MT depolymerization and tubulin degradation. Deletion of both *espG* and its homologue *orf3* revealed a delay in the drop in TER caused by EPEC. The EPEC-induced loss of the MT network is reversible after the infection is eradicated with gentamicin. In addition, the genetically similar pathogen, EHEC, that harbours an EspG homologue (98% identity to EPEC EspG), also disrupted MT in host cells and the *Shigella* homologue *virA* complemented the MT phenotype in an EPEC Δ *espG* strain. Infection with a non-pathogenic *E. coli* strain had no effect on MTs. Dependence of this phenotype on a functional TTSS was consistent with a secreted effector being responsible. In our model system, deletion of *espG* blocked the MT phenotype for up to 6 h. In addition, complementation analysis and stable transfectants expressing EspG further supported a role for EspG in MT disruption in polarized IECs.

Microtubule disruption by EPEC and EHEC was noted first by Li *et al.* (1999) but the effector molecule responsible for this observation was not identified. Recently,

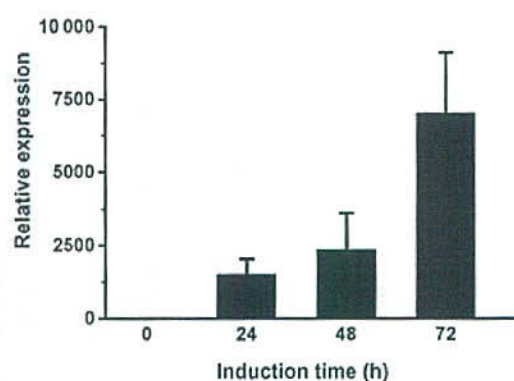
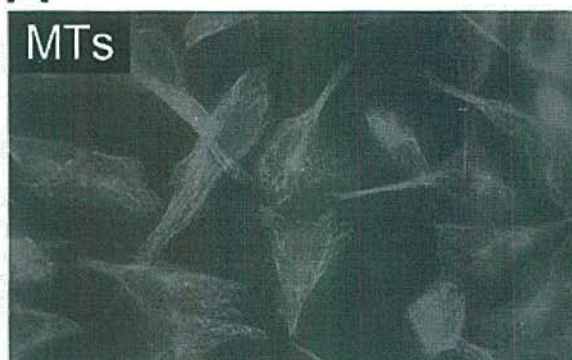


Fig. 8. Relative expression of the *espG* mRNA in HeLa cells transfected with *espG*. Transfected HeLa cells were induced with 2 μ g ml⁻¹ doxycycline for 3 days. RT-PCR analysis of the *espG* mRNA showed an increase in expression over time with maximal expression occurring after 72 h.

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242

A



B



C

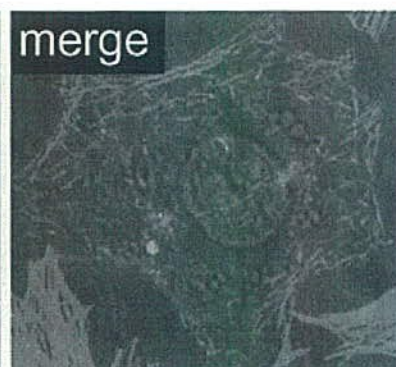
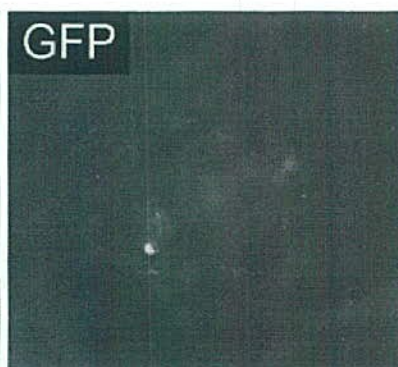
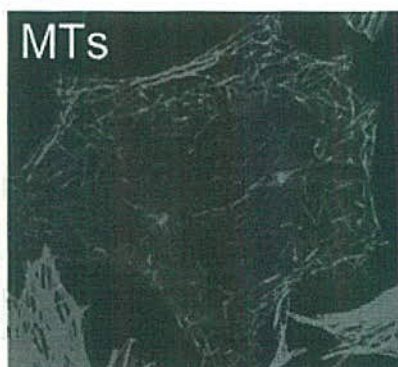


Fig. 9. Expression of EspG in HeLa cells results in MT disruption. Immunofluorescent staining was performed using α -tubulin (red) or EGFP (green) or phase-contrast (grey).

A. Vector control HeLa cells. Note that these HeLa cell monolayers are non-confluent and thus the lack of staining (intermittent blackness) represents the absence of cells, in contrast to the previous figures.

B. HeLa cells transfected with *espG* were induced for 3 days with $2 \mu\text{g ml}^{-1}$ doxycycline. Non-EGFP-expressing cells show an intact MT network with bright MT staining, while those expressing EGFP show fragmented or missing MTs. The corresponding phase-contrast image shows healthy cells. The merged image demonstrates the correspondence of EGFP expression and α -tubulin degradation.

C. High magnification image of a transfected EGFP-expressing HeLa cell showing depolymerized MT fragments. The corresponding EGFP only and merged images are shown.

Matsuzawa *et al.* (2004) showed that both purified GST-tagged EspG and Orf3 depolymerized purified MTs *in vitro*. They also demonstrated that wild-type EPEC and the individual mutants of *espG* and *orf3*, respectively, retained the ability to disrupt MTs in mouse Swiss 3T3 fibroblasts in a localized manner limited to the area directly beneath A/E lesions. Only a double knockout of *espG* and *orf3* prevented localized MT disruption in these cells, suggesting that both Orf3 and EspG are expressed and play redundant roles. In contrast to their studies, we demonstrated that the single mutant, ΔespG , is impaired in its ability to disrupt MTs in polarized epithelial cells for at least 6 h. Moreover, unlike the limited EPEC-induced disruption of MTs reported in fibroblasts or HeLa cells, we saw more dramatic and global destruction of MTs in IECs.

Our initial studies aimed at identifying a functional phenotype for EspG were unrevealing, showing no change in attachment, A/E lesion formation, the inflammatory response or the decrease in TER. We subsequently created a double mutant for both *espG* and *orf3* and tested the effect of this strain on TER, a well-described functional consequence of EPEC infection. To our surprise, mutation of both genes significantly delayed the decrease in TER by EPEC. This is especially intriguing as a ΔespF strain, which is still proficient in EspG/Orf3-dependent MT disruption, is substantially impaired in barrier function alteration (McNamara *et al.*, 2001). Moreover, a $\Delta\text{espF}/\Delta\text{map}$ double mutant was unable to alter barrier function, suggesting that other effector molecules such as EspG and Orf3 probably play only an ancillary role in barrier function alteration (Dean and Kenny, 2004). Our finding that treatment of intestinal epithelial monolayers with neither nocodazole nor paclitaxel altered TER, in contrast to data reported in endothelial monolayers, supports this possibility (Birukova *et al.*, 2004).

The role of EspG and Orf3 in barrier function alteration is unknown at this point; however, several explanations are offered. One potential mechanism involves the reported MT-dependent activation of GEF-H1 and subsequent RhoA stimulation, as observed in Swiss 3T3 (mouse fibroblasts) and transfected, non-polarized epithelial, HeLa, cells. While overexpression of wild-type GEF-H1 in polarized MDCK cells had no effect on stress fibre formation, TJ development, or on TER, permeability to a

small molecule, 4 kDa FITC-dextran, was increased four to fivefold. However, extrapolation of this finding, an increase in permeability to small molecules in the face of structurally normal TJs and unaltered TER, to EPEC pathogenesis is difficult as EPEC induces such dramatic structural and functional changes in TJs (Muza-Moons *et al.*, 2004). While Krendel *et al.* (2002) convincingly demonstrated that MTs play an important role in the regulation of GEF-H1 and RhoA activation, it is important to note that the association of GEF-H1 with cytoskeletal structures varies depending on the cell type examined (Benais-Pont *et al.*, 2003). With the exception of GEF-H1 association with mitotic spindles, GEF-H1 interaction with MTs has been observed only in transfected cells markedly overexpressing this protein (Benais-Pont *et al.*, 2003). In fibroblasts and non-polarized epithelial cells, endogenous GEF-H1 associates with actin stress fibres, not MTs. Of particular relevance to this work is the demonstration that in intestinal epithelial Caco-2 cells, endogenous GEF-H1 colocalizes to F-actin at cell-cell junctions, not to actin stress fibres or MTs. Further, depolymerization of MTs in MDCK cells by nocodazole had no effect on the distribution of endogenous GEF-H1 indicating that the junctional association of GEF-H1 does not depend on MTs. Thus, it is not known whether MT disruption in polarized epithelia activates endogenous GEF-H1 and RhoA. These data complicate the interpretation of experiments performed in fibroblasts and non-polarized epithelial cells regarding EPEC pathogenesis.

Irrespective of the mechanism of activation, RhoA has been demonstrated to play a role in TJ alterations (Jou *et al.*, 1998). For instance, cytotoxic necrotizing factor (CNF)-1, a bacterial toxin that constitutively activates Rho GTPases via inhibition of GTP hydrolysis, disrupted barrier function by displacing occludin and ZO-1 and reorganizing JAM-1 away from the membrane (Hopkins *et al.*, 2003). To directly assess the role of RhoA in EPEC-induced changes in barrier function, we examined the effect of RhoA inhibition on EPEC-induced TER alterations. Unfortunately, recommended concentrations of the Rho-kinase inhibitor Y27632 had a profound impact on the TER of established intestinal epithelial (our unpublished data) and MDCK monolayers (Benais-Pont *et al.*, 2003) thus prohibiting the performance of these key experiments.

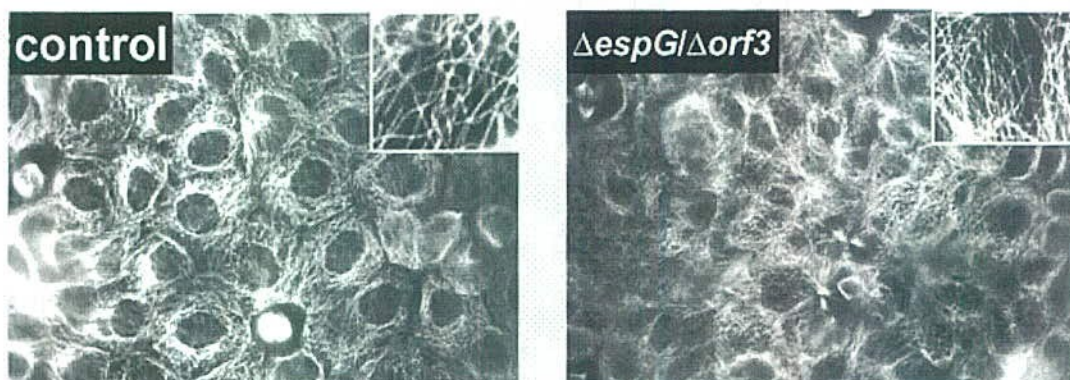
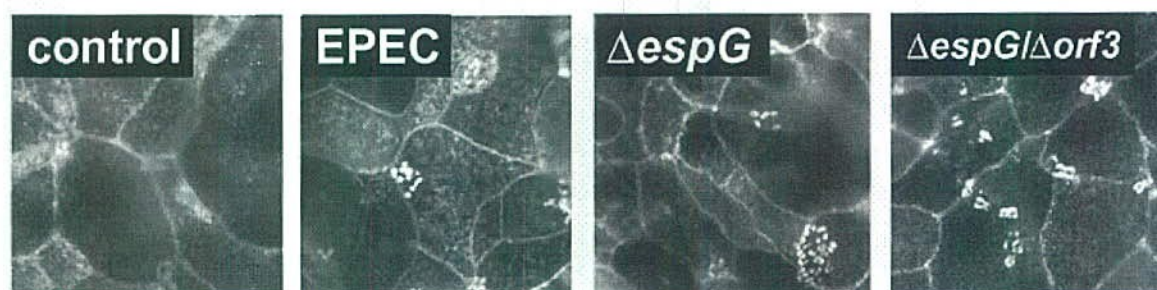
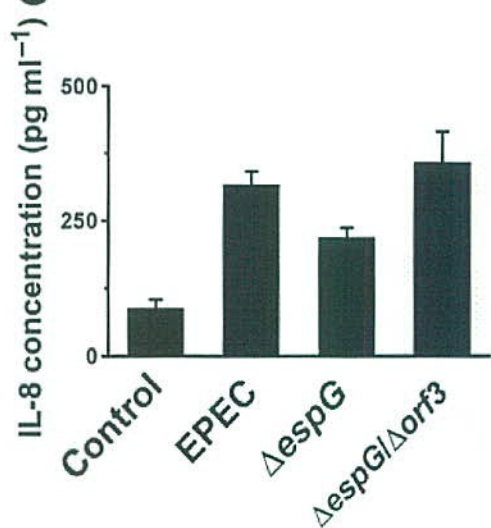
A**B****C**

Fig. 10. Effect of $\Delta espG$ versus $\Delta espG/\Delta orf3$ on host cell phenotypes.

A. Immunofluorescent staining for α -tubulin was performed on control Caco-2 monolayers and those infected with $\Delta espG/\Delta orf3$ for 4 h. The MT network of monolayers infected with $\Delta espG/\Delta orf3$ was intact and similar to that of control monolayers. The insets highlight individual MT fibres.

B. Immunofluorescent staining for actin, using phalloidin-conjugated FITC, showed the appearance of actin pedestals at 2 h for wild-type EPEC, $\Delta espG$ and $\Delta espG/\Delta orf3$.

C. IL-8 secretion by HT-29 cells showed that all three strains produced an inflammatory response compared with uninfected cells. IL-8 secretion in response to $\Delta espG$ was slightly but significantly less than in response to wild-type EPEC ($P = 0.01$), while that after infection with the double mutant was no different from wild-type EPEC ($P = 0.52$). Data represent mean $\pm$ SEM ($n = 6$ for each group).

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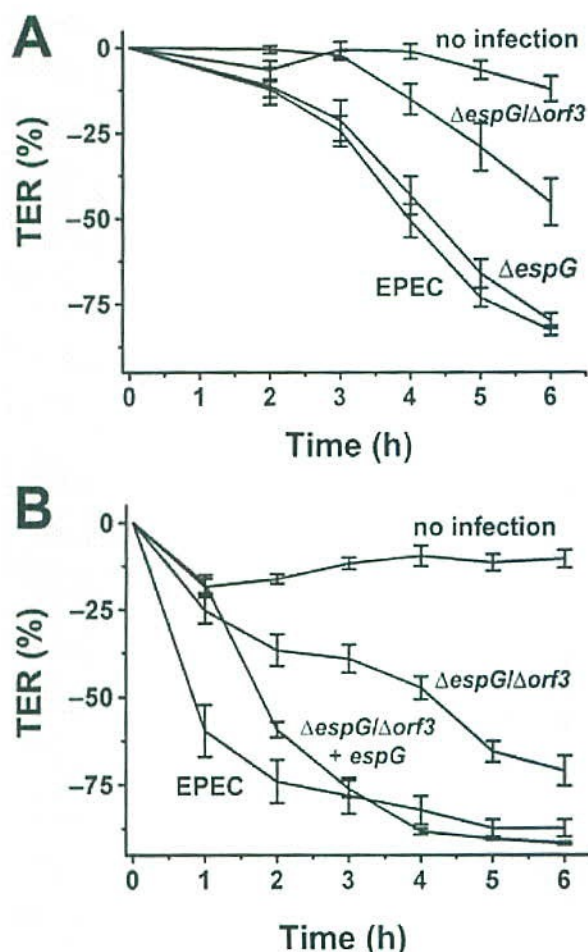


Fig. 11. Deletion of *espG* and *orf3* delays the drop in TER by EPEC. **A.** TER was measured at hourly time intervals after infection of T84 monolayers with EPEC. As can be seen, deletion of both *espG* and *orf3*, but not of *espG* alone, significantly delayed the EPEC-induced decrease in TER. ($n = 7-19$ for each group; $P < 0.05$ for $\Delta espG/\Delta orf3$ versus wild-type EPEC after 6 h of infection). **B.** Infection of Caco-2 monolayers showed a similar trend of delayed decrease in TER after infection with the double mutant, but not that harbouring a mutation in *espG* alone ($n = 6-12$ for each group; $P = 6.3 \times 10^{-7}$ for $\Delta espG/\Delta orf3$ versus wild-type EPEC after 6 h of infection). Complementation of $\Delta espG/\Delta orf3$ with *espG* restored the wild-type TER phenotype.

Clearly, the disruption of TJs by EPEC is complex involving at least two effector molecules, EspF and Map, and this work suggests perhaps others. How these effectors co-ordinate to bring about TJ disruption is so far unsolved. The regulation of TJ barrier function is very complex with several transmembrane and intracellular proteins being involved. The mechanisms underlying this physiological alteration, however, are worthy of pursuit in that TJ perturbation is now recognized as a characteristic of several disease states including not only enteric infection but Crohn's disease, magnesium deficiency, possibly asthma and others (Hecht, 2003; Sawada *et al.*, 2003).

It is well known that MTs are required for the establishment and maintenance of epithelial cell polarity. Interestingly, unlike fibroblasts, polarized epithelial cells do not require MTs to maintain their shape (Middleton *et al.*, 1988). While MTs may not interact directly with TJs, they do position organelles including the Golgi and ER (Ku *et al.*, 1999). We have reported previously that EPEC infection disrupts not only TJ barrier function but also fence function, or the maintenance of cell polarity, as well which probably contributes to EPEC pathogenesis by permitting the redistribution of $\beta 1$ integrin to the apical membrane thus providing an alternate receptor for intimin (Muza-Moons *et al.*, 2003). Whether this results from disruption of TJs or altered positioning of cytoplasmic organelles is unknown nor is the dependence of this phenotype on EspG/Orf3. Alternatively, MTs also play a role in membrane and receptor recycling (Knight *et al.*, 1995; Ku *et al.*, 1999). Therefore, EPEC destruction of the host MT network may actually prevent membrane and receptor recycling, which would allow Tir to remain in the apical membrane for prolonged periods.

In order to discern the contributions of these alterations to EPEC pathogenesis, studies with *espG* and *orf3* mutant strains will need to be performed in animal models. We have recently reported that the C57BL/6 mouse provides a model of human EPEC infection, at least as regards the inflammatory phenotype (Savkovic *et al.*, 2005). It will be useful to determine whether the effects of EPEC on the TJ permeability and polarity of intestinal epithelia *in vivo* parallel findings previously reported *in vitro* (Simonovic *et al.*, 2000; 2001; McNamara *et al.*, 2001; Muza-Moons *et al.*, 2003; Dean and Kenny, 2004).

In summary, we have shown here that EPEC EspG induces the fragmentation of MTs and the degradation of tubulin in IECs. This phenotype is dependent on a functional TTSS and expression of EspG. Expression of EspG in HeLa cells was sufficient for production of this phenotype. While deletion of *espG* alone had no apparent physiological phenotype, mutation of *espG* and its homologue *orf3* caused a significant delay in the disruption of the TJ barrier. Whether EspG/Orf3 are essential for inducing disease in humans infected with EPEC is not known; however, these proteins clearly have major effects on the cytoskeletal composition of intestinal epithelial host cells and probably interfere with physiological functions thus warranting further investigation.

Experimental procedures

Cell culture

T84, HeLa, HT-29 and Caco-2 polarized human IECs were used in these studies. T84 cells were grown in a 1:1 (v/v) mixture of Dulbecco-Vogt modified Eagle medium (D-MEM) (Invitrogen, Carlsbad, CA) and Ham's F-12 (Invitrogen) with

6% newborn calf serum (Invitrogen) at 37°C in 5% CO₂ as previously described (Madara *et al.*, 1987; Simonovic *et al.*, 2000). HT-29 and Caco-2 cells were grown in high glucose D-MEM with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C in 5% CO₂. Transfected HeLa cells were grown in D-MEM with 10% tetracycline-free FBS (BD Biosciences, San Jose, CA), supplemented with 100 µg ml⁻¹ Geneticin (G418) (Sigma, St Louis, MO), 100 units ml⁻¹ penicillin G (Sigma) and 100 µg ml⁻¹ streptomycin sulphate (Invitrogen) at 37°C in 5% CO₂. The *espG* stable transfectants were similarly maintained with the addition of 100 µg ml⁻¹ hygromycin B (BD Biosciences).

Plasmids and strains: construction of EPEC strains

EPEC mutant construction was previously described (Elliott *et al.*, 2001). Primers K204 (5'-AAATGCATGTCTGTATTC CAAT) and K707 (5'-ATGAGGAATTCACACCTATGCAG TCG) were used to create E2348/69 Δ map which was designated SE882. For complementation experiments, *S. flexneri* 2a (strain 2457T) *virA* was PCR amplified using primers VirA-1 (5'-ATGCAGACATCAAACATAAC) and VirA-2 (5'-AACATCAGGAGATATGATGGC) and cloned into the pGEM[®]-T Easy Vector (Promega, Madison, WI). The resulting plasmid, pRPK16, was transformed into Δ espG to make strain GH292. EPEC *espG* was PCR amplified using primers EspG-3 (5'-CACCATGGTACTTGTTGCCAAATTG) and EspG-4 (5'-AGTGTTTTGTAGTACGTTTCAG), and the resulting fragment was cloned into pTrc2HisTOPO (Invitrogen). After verifying the orientation of the insert, the *NcoI* fragment at the 5' end was eliminated to locate the gene at the optimal distance from the ribosome binding site. The resulting plasmid, pVK202, was transformed into the Δ espG Δ orf3 strain, and used in complementation studies in the presence of 1 mM IPTG. For transfection studies, *espG* was PCR amplified with Taq DNA polymerase (Invitrogen)

using the primers K2631 (5'-ACGACGCGTTGATGCTATAT GATACTTGTG) and K2632 (5'-CTAGCTAGCTGGGTGT GATTCTATTATTGG), and the *MluI*-*NheI* fragment was cloned into the corresponding sites in the tetracycline-responsive EGFP expression vector pBI-EGFP (BD Biosciences). Plasmids were verified by PCR, restriction digests and, where required, sequence analysis.

Growth of bacteria and infection of host cells

The specific strains used in these studies are listed in Table 1. Either E2348/69 or JPN15/pMAR7, an ampicillin-resistant derivative of E2348/69, was used as the wild-type EPEC in all experiments (Baldini *et al.*, 1983). All strains were grown overnight in LB with antibiotics and diluted 1:33 in serum-free T84 media and grown to the optical density (OD) reading of 0.3. T84 or Caco-2 monolayers were infected with EPEC at an moi of ~50. After 1 h, medium was aspirated and replaced, and monolayers were incubated for additional time periods as indicated (Savkovic *et al.*, 1997; Yuhan *et al.*, 1997; Hecht *et al.*, 1999).

Electrophysiological studies

The measurement of TER was performed as previously described (Madara *et al.*, 1992; Yuhan *et al.*, 1997).

Detection of IL-8

HT-29 cells were grown in 24-well plates. The monolayers were infected with 200 µl of EPEC at an moi of ~50. After 1 h, medium was added to a final volume of 1 ml. IL-8 was determined using a Quantikine IL-8 immunoassay kit (R&D Systems, Minneapolis, MN) from samples taken at 6 h after infection.

Table 1. Strains used in this study.

Strain	Description	Reference or source
EPEC		
JPN15/pMAR7	E2348/69/Amp ^R EAF	Baldini <i>et al.</i> (1983)
E2348/69	EPEC O127:H7	Knutton <i>et al.</i> (1989)
UMD864	E2348/69 <i>espB</i>	Donnenberg <i>et al.</i> (1993)
UMD872	E2348/69 <i>espA</i>	Kenny <i>et al.</i> (1996)
UMD870	E2348/69 <i>espD</i>	Lai <i>et al.</i> (1997)
KC14	JPN15/pMAR7 <i>tir</i>	Campellone <i>et al.</i> (2002)
CVD206	E2348/69 <i>aeA</i>	Donnenberg and Kaper (1991)
UMD874	E2348/69 <i>espF</i>	McNamara and Donnenberg (1998)
SE882	E2348/69 <i>map</i>	This work
CVD452	E2348/69 <i>escN</i>	Jarvis <i>et al.</i> (1995)
Δ espG	E2348/69 <i>espG</i>	Elliott <i>et al.</i> (2001)
Δ espG/pCVD453	<i>espG</i> complemented	Elliott <i>et al.</i> (2001)
SE1114	E2348/69 <i>espG</i> /pUC18	This work
Δ espG/ Δ orf3	E2348/69 <i>espG</i> and <i>orf3</i>	Elliott <i>et al.</i> (2001)
GH292	Δ espG/pRPK16 <i>virA</i> complemented	This work
GH313	Δ espG/ Δ orf3/pVK202 <i>espG</i> complemented	This work
EHEC		
85-170	EHEC O157:H7 stx-	Jarvis and Kaper (1996)
Other		
HS-4	O9:H14 commensal <i>E. coli</i>	Jarvis and Kaper (1996)

Immunofluorescence microscopy

Control and infected monolayers of T₈₄ and Caco-2 cells were fixed on glass coverslips using the 'DSP-Tsb' protocol, which preserves MTs and allows actin staining using conjugated phalloidin (Bell and Safiejko-Mroczka, 1995; Nakamura, 2001). Briefly, coverslips were washed for 5 min in PBS followed by a 10 min incubation in 1 mM DSP [dithiobis (succinimidylpropionate)] in Hanks Balanced Salt Solution (HBSS) (Invitrogen), 10 min in 1 mM DSP in Tsb [0.5% Triton X-100 in MTSB (microtubule stabilizing buffer: 1 mM EGTA, 4% PEG 8000, 100 mM PIPES pH = 6.9)], 5 min in Tsb, 15 min in 4% paraformaldehyde in MTSB, 5 min in PBS, 5 min in 0.1 M glycine-PBS, and finally, 5 min in PBS. Cells were incubated with Zymed blocking solution (Zymed, San Francisco, CA) for 1 h and then with primary antibody against α -tubulin (clone B-5-1-2) (Sigma) overnight followed by Alexa Fluor 568- or Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 h. Staining with a β -tubulin antibody yielded similar staining (Zymed). Fluorescein phalloidin (Molecular Probes) was used to visualize actin. Monolayers were washed and mounted on glass microscope slides with ProLong Antifade reagent (Molecular Probes). Stained monolayers were visualized and photographed using a Nikon Opti-Phot inverted microscope equipped with the Spot-RT Digital Imaging System (Diagnostic Instruments, Sterling Heights, MI).

Protein extraction and immunoblotting

Protein was extracted in a modified RIPA buffer (20 mM Tris HCl pH = 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 25 μ g ml⁻¹ leupeptin and 100 μ M paclitaxel) as previously described (Simonovic *et al.*, 2001), resolved by 12% SDS-PAGE and analysed by immunoblotting. The proteins from the 12% SDS-PAGE were transferred onto 0.45 μ m pore-size nitrocellulose membranes using a Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA) and analysed by immunoblotting (Simonovic *et al.*, 2001).

Construction of HeLa-Tet-EspG cell line

For transfection quality plasmid DNA, plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen). pBI-EspG and the transfection selection vector pTK-Hyg (BD Biosciences) were co-transfected into the HeLa Tet-On cell line (BD Biosciences) using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturers' instructions. The HeLa Tet-On cell line expresses the reverse tetracycline-controlled transactivator (rtTA), which is able, upon the addition of tetracycline or doxycycline, to activate expression of EGFP and a gene of interest (in this case *espG*) from the bidirectional Tet-responsive promoter present in pBI-EGFP derivatives. Stable transfectants were selected in DMEM (Invitrogen)/10% FBS (BD Biosciences)/200 μ g ml⁻¹ hygromycin (BD Biosciences) and screened by observance of doxycycline, 2 μ g ml⁻¹ (BD Biosciences)-inducible EGFP expression. Doxycycline-inducible expression of *espG* in the resulting HeLa-Tet-EspG cell line was confirmed by RT-PCR.

The cell line was expanded and aliquots were stocked in storage medium (70% FBS, 20% DMEM, 10% DMSO).

Real-time PCR assessment of EspG induction

HeLa-Tet-EspG cells were plated at approximately 50% confluence in six-well tissue culture plates (Corning, NY) containing DMEM, 10% FBS and 2 μ g ml⁻¹ doxycycline. Samples were incubated at normal tissue culture conditions and the above medium was replaced every 24 h. Three independent RNA samples were isolated at 0, 24, 48 and 72 h after the start of induction. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and resuspended in The RNA Storage Solution (Ambion, Austin, TX). RNA quality was determined by spectrophotometry and electrophoresis on a 1% agarose gel. RNA (5 μ g) was treated with DNase I (Invitrogen) at a concentration of 1 U μ g⁻¹ RNA. DNase I-treated RNA (2 μ g) was subjected to first-strand complementary DNA synthesis using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using QuantiTect™ SYBR® Green PCR Kit (Qiagen) reagents. One microlitre of a 1:10 dilution of cDNA, 900 nM of each primer (EspG-F: TCGAGGACTCGGCAATGCA and EspG-R: AACATTCCGGGCAAGTGATGA, or GAPDH-F: GATCATCAGCAATGCCTCCT and GAPDH-R: TGTGGTCATGAGTCCTTCCA), and 12.5 μ l of SYBR® Green master mix were included in a 25 μ l final reaction volume. All samples were tested in triplicate. Amplification conditions were as follows: 1 cycle (95°C for 10 min), 40 cycles (95°C for 15 s followed by 60°C for 1 min). A melting curve analysis was performed by heating to 95°C for 15 s, cooling to 60°C, then slowly heating to 95°C while monitoring fluorescence, to confirm the amplification of one specific product. No contamination by genomic DNA was detectable by PCR of DNase I-treated RNA.

Data were collected using ABI PRISM® SDS 2.2 software (Applied Biosystems). EspG levels were normalized to levels of GAPDH and analysed using the comparative CT method as described (1997, Applied Biosystems, User Bulletin #2 Perkin Elmer). Relative expression comparisons were made between the 0 h time point and subsequent time points. Data are presented as the geometric mean, with error bars representing standard deviation of the mean. (Note: as the 0 h time point was used as the baseline for comparison it is set to 1.)

Statistical analysis

Data were analysed using Student's *t*-test for independent samples. Differences were considered significant if *P* was ≤ 0.05 .

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