

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
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DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	378 FASE NACIONAL INCLUIDO CAPITULO I
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	7
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 11-68894- -10
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 3082
 Folios: 7

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2009/066109				
Fecha de Presentación Internacional	01/12/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 11-068894-00000-0000	2/Junio/2011
Reivindicaciones:	Radicado No. 11-068894-00000-0000	2/Junio/2011
	Radicado No. 11-068894-00009-0000	4/Junio/2013
Respuesta del solicitante:	Radicado No. 11-068894-00009-0000	4/Junio/2013
Prioridad:	EP 08170591.5	3/Diciembre/2008

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Radicado No. 11-068894-00009-0000) porque no se amplía el objeto de la solicitud.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso, se tiene en cuenta la totalidad del nuevo capítulo reivindicatorio (Radicado No. 11-068894-00009-0000) para el examen de patentabilidad.

Título de la solicitud: "PROCEDIMIENTO PARA LA PRODUCCIÓN DE VACUNAS"

El problema planteado en esta solicitud consiste en que la producción actual de vacunas contra patógenos bacterianos que producen toxinas AB se realiza mediante la inactivación química de las mismas, o mediante producción de mutantes atóxicos. Sin embargo, éstas medidas conducen a una pérdida de epítomos antigénicos del organismo patógeno que puede afectar a la eficacia de la vacuna.

Para resolver este problema técnico la solicitud en estudio proporciona un procedimiento para la producción de vacunas contra patógenos bacterianos que producen toxina AB, el cual inactiva las toxinas AB mediante un proceso enzimático autocatalítico *in vitro* que aprovecha la actividad proteolítica intrínseca de tales toxinas.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/08; C07K 14/33.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 a 7 carecen de concisión porque al tratarse de un procedimiento para la producción de una vacuna, no se definen las condiciones en que se llevan a cabo las etapas de (a) cultivar el patógeno, (b) escindir la toxina y (c) combinar la composición. Por ejemplo, no se definen las condiciones de cultivo del patógeno que permiten que éste produzca la toxina AB, así como temperatura, medio de cultivo, concentración de fosfato de inositol para escindir la toxina AB, entre otros. Igualmente se utilizan expresiones como "patógenos bacterianos", "toxina AB", "vehículo farmacéuticamente aceptable", lo cual dificulta determinar la extensión del objeto que se busca proteger. Se sugiere definir el tipo de patógeno, la toxina AB por su secuencia de aminoácidos y el vehículo por sus componentes.

La reivindicación 5 reclama un procedimiento donde el patógeno es de géneros como *Clostridium*, *Vibrio*, *Bordetella*, entre otros. Sin embargo, este Despacho encuentra que solamente se encuentra ejemplificado en la descripción un proceso que utiliza *Clostridium difficile*, de manera que no existe evidencia que el proceso enzimático autocatalítico pueda ser aplicado con otros patógenos. Se sugiere hacer la aclaración respectiva.

Las reivindicaciones 8 a 10 caracterizan la vacuna por su proceso de fabricación. Se recuerda a la solicitante que las reivindicaciones de productos definidas en términos de un proceso de fabricación son admisibles, solamente si los productos como tales cumplen los requisitos de patentabilidad, es decir, cuando son nuevos e inventivos, y cuando no se pueden definir por sus características estructurales. En este caso, la vacuna, por tratarse de una composición, se caracteriza por sus componentes y proporciones dentro de la misma. En el caso de los componentes, el toxoide se caracteriza por su secuencia de aminoácidos.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 3 de diciembre de 2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D2	Nature, Vol. 446, Págs. 415-419	"Autocatalytic cleavage of Clostridium difficile toxin B"	22/Marzo/2007
D3	Immunology and Cell Biology, Vol. 82, Págs. 488-496	"Vaccine adjuvants: current state and future trends"	28/Septiembre/2004
D4	Infection and Immunity, Vol. 69, Págs. 988-995	"Safety and immunogenicity of increasing doses of a Clostridium difficile toxoid vaccine administered to healthy adults"	Febrero/2001

D2¹ divulga Un procedimiento para la producción de una toxina AB en un vehículo farmacéuticamente aceptable (Pág. 417, leyenda de la Fig. 2 y Pág. 418).

D3² enseña Adyuvantes para vacunas como el MPLA (Resumen y Pág. 490).

D4³ divulga una vacuna que comprende el Toxoide A y el Toxoide B de Clostridium difficile (Resumen).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un procedimiento para la producción de una vacuna contra patógenos bacterianos que producen una toxina AB, que comprende

- (a) cultivar el patógeno en condiciones en las que se produce la toxina AB, y recoger el cultivo;
- (b) escindir la toxina AB enzimáticamente *in vitro*, en donde se usa fosfato de inositol como cofactor de la escisión enzimática; y
- (c) combinar la composición de la etapa (b) con un vehículo farmacéuticamente aceptable.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2
Un procedimiento para la producción de una vacuna contra patógenos bacterianos que producen una toxina AB, que comprende:	Un procedimiento para la producción de una toxina AB que comprende:

1 <http://www.ncbi.nlm.nih.gov/pubmed/17334356>

2 <http://www.nature.com/icb/journal/v82/n5/full/icb200475a.html>

3 <http://www.ncbi.nlm.nih.gov/pubmed/11159994>

(a) cultivar el patógeno en condiciones en las que se produce la toxina AB, y recoger el cultivo;	a) Cultivar el patógeno en condiciones en las que se produce la toxina AB (Pág. 418, Col. izquierda, Párr. 7)
(b) escindir la toxina AB enzimáticamente <i>in vitro</i> , en donde se usa fosfato de inositol como cofactor de la escisión enzimática; y	b) escindir la toxina AB enzimáticamente <i>in vitro</i> , en donde se usa fosfato de inositol como cofactor de la escisión enzimática (Pág. 418, Col. derecha, Párr. 2)
(c) combinar la composición de la etapa (b) con un vehículo farmacéuticamente aceptable.	c) combinar la composición de la etapa (b) con un vehículo farmacéuticamente aceptable. (Pág. 417, leyenda de la Fig. 2)

Las características esenciales del procedimiento de la reivindicación 1 habían sido divulgadas previamente en el documento D2, el cual enseña un procedimiento para la producción de una toxina AB que comprende: a) cultivar el patógeno en condiciones en las que se produce la toxina AB (Pág. 418, Col. izquierda, Párr. 7); b) escindir la toxina AB enzimáticamente *in vitro*, en donde se usa fosfato de inositol como cofactor de la escisión enzimática (Pág. 418, Col. derecha, Párr. 2) y c) combinar la composición de la etapa (a) con un vehículo farmacéuticamente aceptable (Pág. 417, leyenda de la Fig. 2). El documento D2 también enseña que la toxina A se purifica a partir de la toxina B antes de la escisión (Pág. 418, Col. derecha, Párr. 1).

En consecuencia, la reivindicación 1 y las reivindicaciones 2 a 6 y 8 carecen de novedad, según lo divulgado en el documento D2.

Nivel inventivo (Art18 D486)

La reivindicación 7 consiste en el procedimiento de las reivindicaciones 1 a 6, en donde se añade un adyuvante a la composición.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
El procedimiento de las reivindicaciones 1 a 6, en donde se añade un adyuvante a la composición.	Un procedimiento para la producción de una toxina AB en un vehículo farmacéuticamente aceptable. (Pág. 417, leyenda de la Fig. 2 y Pág. 418).	Adyuvantes para vacunas como el MPLA. (Resumen y Pág. 490)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 7 porque enseña un procedimiento para la producción de una toxina AB que comprende: a) cultivar el patógeno en condiciones en las que se produce la toxina AB (Pág. 418, Col. izquierda, Párr. 7); b) escindir la toxina AB enzimáticamente *in vitro*, en donde se usa fosfato de inositol como cofactor de la escisión enzimática (Pág. 418, Col. derecha, Párr. 2) y c) combinar la composición de la etapa (a) con un vehículo farmacéuticamente aceptable (Pág. 417, leyenda de la Fig. 2).

La diferencia entre la invención, definida en la reivindicación 7 y el procedimiento del documento D2 consiste en que se añade un adyuvante a la composición.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja al procedimiento reclamado en esta solicitud porque no permite obtener una

composición más inmunogénica.

El problema técnico objetivo de esta solicitud consiste en la necesidad de un procedimiento para preparar una vacuna contra patógenos bacterianos que producen una toxina AB alternativa a la ya conocida en el documento D2.

Teniendo en cuenta que el documento D2 ya divulgaba un procedimiento para la producción de una toxina AB en un vehículo farmacéuticamente aceptable (Pág. 417, leyenda de la Fig. 2 y Pág. 418) y que el documento D3 enseña adyuvantes para vacunas, dentro de los cuales se encuentra el MPLA (Resumen y Pág. 490), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, incluir un adyuvante en la composición producida por el procedimiento del documento D2, para llegar así al objeto de la reivindicación 7.

De manera que la solución propuesta por esta solicitud en la reivindicación 7 es obvia y no tiene nivel inventivo.

La reivindicación 9 consiste en la vacuna de la reivindicación 8, que comprende toxoide A y/o Toxoide B de *Clostridium difficile*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D4
La vacuna de la reivindicación 8, que comprende toxoide A y/o Toxoide B de <i>Clostridium difficile</i> .	Una composición que comprende el Toxoide B de <i>Clostridium difficile</i> producido por escisión enzimática. (Pág. 418, Col. derecha, Párr. 2)	Una vacuna que comprende Toxoide A y Toxoide B de <i>Clostridium difficile</i> . (Resumen)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 9 porque enseña una composición que comprende el Toxoide B de *Clostridium difficile* producido por escisión enzimática (Pág. 418, Col. derecha, Párr. 2).

La diferencia entre la invención, definida en la reivindicación 9 y la composición del documento D2 consiste en que también puede comprender el Toxoide A de *Clostridium difficile*.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la vacuna reclamada en esta solicitud porque no es más inmunogénica, según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una composición alternativa a la ya conocida en el documento D2.

Teniendo en cuenta que el documento D2 ya divulgaba una composición que comprende el Toxoide B de *Clostridium difficile* producido por escisión enzimática (Pág. 418, Col. derecha, Párr. 2) y que el documento D4 enseña una vacuna que comprende el Toxoide A y el Toxoide B de *Clostridium difficile* (Resumen), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, utilizar el procedimiento divulgado en el documento D2 para escindir enzimáticamente el Toxoide A y el Toxoide B de *Clostridium difficile*, teniendo en cuenta que son homólogos entre sí (Pág. 415, Col. izquierda, Párr. 2), para llegar así al objeto de la reivindicación 9.

De manera que la solución propuesta por esta solicitud en la reivindicación 9 y en la reivindicación 10 es obvia y no tiene nivel inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	Nature, Vol. 446, Págs. 415-419	1 – 10	Novedad y Nivel inventivo
D3	Immunology and Cell Biology, Vol. 82, Págs. 488-496	7	Nivel inventivo
D4	Infection and Immunity, Vol. 69, Págs. 988-995	9 y 10	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-03-11

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Autocatalytic cleavage of *Clostridium difficile* toxin B

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Clostridium difficile, the causative agent of nosocomial antibiotic-associated diarrhoea and pseudomembranous colitis, possesses two main virulence factors: the large clostridial cytotoxins A and B. It has been proposed that toxin B is cleaved by a cytosolic factor of the eukaryotic target cell during its cellular uptake. Here we report that cleavage of not only toxin B, but also all other large clostridial cytotoxins, is an autocatalytic process dependent on host cytosolic inositolphosphate cofactors. A covalent inhibitor of aspartate proteases, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, completely blocked toxin B function on cultured cells and was used to identify its catalytically active protease site. To our knowledge this is the first report on a bacterial toxin that uses eukaryotic signals for induced autoproteolysis to deliver its toxic domain into the cytosol of target cells. On the basis of our data, we present an integrated model for the uptake and inositolphosphate-induced activation of toxin B.

With the introduction of antibiotic therapy into clinical practice the lifespan of the population in the industrialized world increased and infections seemed to become manageable. However, this observation is deceptive, because many microbes possess or develop resistance mechanisms^{1,2}. Furthermore, antibiotics may cause prominent side effects, like the observed increase of pseudomembranous colitis when clindamycin was introduced in the 1970s^{3,4}. Today, we know that nearly all known antibiotics are capable of inducing antibiotic-associated diarrhoea or its fatal form, pseudomembranous colitis^{5,6}.

The factors inducing antibiotic-associated diarrhoea or pseudomembranous colitis are two toxins produced by *Clostridium difficile*, toxin A (TcdA) and toxin B (TcdB)^{7,8}, which are homologous to each other⁹, to TcsH and TcsL of *Clostridium sordellii*¹⁰ and to Tcn α of *Clostridium novyi*¹¹. Because of their sequence homology, similar domain structure and glycosyltransferase properties these toxins are designated 'large clostridial cytotoxins' (LCTs)^{12,13}.

Recently, *C. difficile* attracted great attention^{14,15} because hyper-virulent, ribotype 027 isolates, which are resistant to fluoroquinolones, were reported in Canada and the USA. These reports and some recent data of younger people encountering severe *C. difficile* disease without prior antibiotic treatment indicate a changing epidemiology and call for a shift in the perception that only elderly, immunocompromised people are at high risk of suffering from *C. difficile* infection, mainly following hospitalization¹⁶. There is the threat that such strains will spread worldwide and thus compromise antibiotic treatment in general^{17,18}. Accordingly, a better understanding of the mode of action of TcdA and TcdB is urgently needed.

TcdA and TcdB are single-chained and have a tripartite functional organization¹²: the carboxy-terminal domain is responsible for binding to the host cell membrane¹⁹; the hydrophobic middle part is supposed to be involved in translocation across cellular membranes²⁰; and the amino-terminal domain harbours the catalytically active glucosyltransferase site^{12,21}. Although knowledge about the uptake process is still fragmentary, it is generally accepted that on

binding to host cell receptors^{22,23} the toxins are endocytosed²⁴. The translocation of the toxins to the cytosol occurs from early endosomal compartments^{25,26}. Because TcdA forms pores in artificial membranes at low pH (ref. 27), it was suggested that in living cells the low endosomal pH triggers similar conformational changes and accordingly may propagate translocation via pore formation.

From early on, it was postulated that the toxin is activated through catalytic processing^{26,28}. Recently, it was shown that TcdB is cleaved between Leu 543 and Gly 544 of TcdB at neutral pH (ref. 29), generating fragments of 63 kDa (catalytic-DXD domain) and 207 kDa (ligand-translocation domain). Only the smaller N-terminal fragment harbouring the glucosyltransferase activity reaches the cytosol, whereas the 207 kDa C-terminal fragment remains in the membrane fraction^{20,29}. Microinjection experiments showed that the 63 kDa fragment exhibits full cytotoxic activity shortly after injection, whereas the holotoxin starts with low activity, which increases with time²⁹, indicating the biologic relevance of proteolytic processing for catalytic activity. The released cytotoxic fragment can then act on its cytosolic targets—the GTPases of the Rho/Rac family^{30,31}. Until now, the assumption was that this cleavage is catalysed by a pepstatin A-sensitive host cell protease^{20,29}.

Results

Identification of the host factor. To identify the host factor required for cleavage of TcdB, we used TcdB preparations³² that were Cy3-labelled as described in the literature²⁹. Cytosolic extracts of porcine splenocytes showed strong cleavage activity (Fig. 1a) and thus served as raw material for further analysis. Purification of cytosolic fractions of splenocyte extracts (Supplementary Fig. 1) led to the following inconsistencies with the thesis that we had purified a cellular protease: concentration by ammonium sulphate precipitation was not possible, activity was fully retained in heated extracts (96 °C, 30 min), effective TcdB cleavage was induced with the aqueous protein-free phase of phenol chloroform extractions and highly purified active

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extracts showed no significant absorption at 280 nm (Supplementary Fig. 1).

The active components were identified by nano-electrospray-ionization quadrupole time-of-flight (nanoESI-QTOF) mass spectrometry of purified active fractions (Fig. 1b). The comparison of fragment spectra of the active substance (Fig. 1c, d) with published spectra³³ revealed strong similarities to inositolphosphates (Fig. 1e). Several inositolphosphates (InsPs) were tested in the cleavage assay and inositolhexaphosphate (InsP₆) showed the highest cleavage activity (see Supplementary Table). Unlabelled TcdB visualized by zinc staining³⁴ showed activity of InsP₆ down to 1 μ M (Supplementary Fig. 2). Because the total cellular InsP₆ concentration is in the range of 15–100 μ M (ref. 35), concentrations of InsP₆ required for *in vitro* cleavage can be regarded as physiologically relevant. No cleavage activity has ever been observed or attributed to InsPs itself. Whereas activation of proteins by phosphorylation is a well known mechanism, inositolphosphates, in contrast to inositolpyrophosphates³⁶, do not contain high energy pyrophosphate bonds. It is therefore unlikely that inositolphosphates activate the toxin by phosphorylation.

Exclusion of contaminant proteases. In accordance with previous publications²⁹, cleavage was pepstatin A-sensitive, indicating that the TcdB preparation contained a latent, InsP_x-inducible aspartate protease (data not shown). A series of experiments was performed

to exclude the contribution of a contaminating InsP_x-inducible protease that might co-purify with TcdB. A putative unspecific action of our TcdB preparation on other proteins was excluded (Supplementary Fig. 3). Using monoclonal antibody 2CV (ref. 37), TcdB was additionally affinity purified, which yielded more than 98% pure TcdB as assessed by SDS–polyacrylamide gel electrophoresis (PAGE). The remaining contaminant proteins were identified by tandem mass spectrometry (MS/MS) analysis. All proteins matched to sequences from the recently published *C. difficile* genome³⁸ and showed no similarity to known endoproteases (data not shown). The affinity-purified TcdB retained the InsP₆-inducible cleavage activity. Further, cleavage of TcdB by a contaminant protease would be an intermolecular process, whereas autocatalysis is intramolecular. Because dilution favours intramolecular reactions in comparison to intermolecular reactions, we performed cleavage of TcdB at different concentrations and observed that in contrast to the partial cleavage at 5 μ M TcdB, cleavage is nearly completed after 30 min at a concentration of 50 nM TcdB (Fig. 2a). This result is inconsistent with the participation of a contaminating protease, which would show reduced activity at lower concentrations. Consequently, we postulated autocatalytic cleavage by an intrinsic TcdB aspartate protease.

Proof of autocatalytic TcdB cleavage. To prove further our model for autocatalytic TcdB activation, we decided to inactivate and identify the

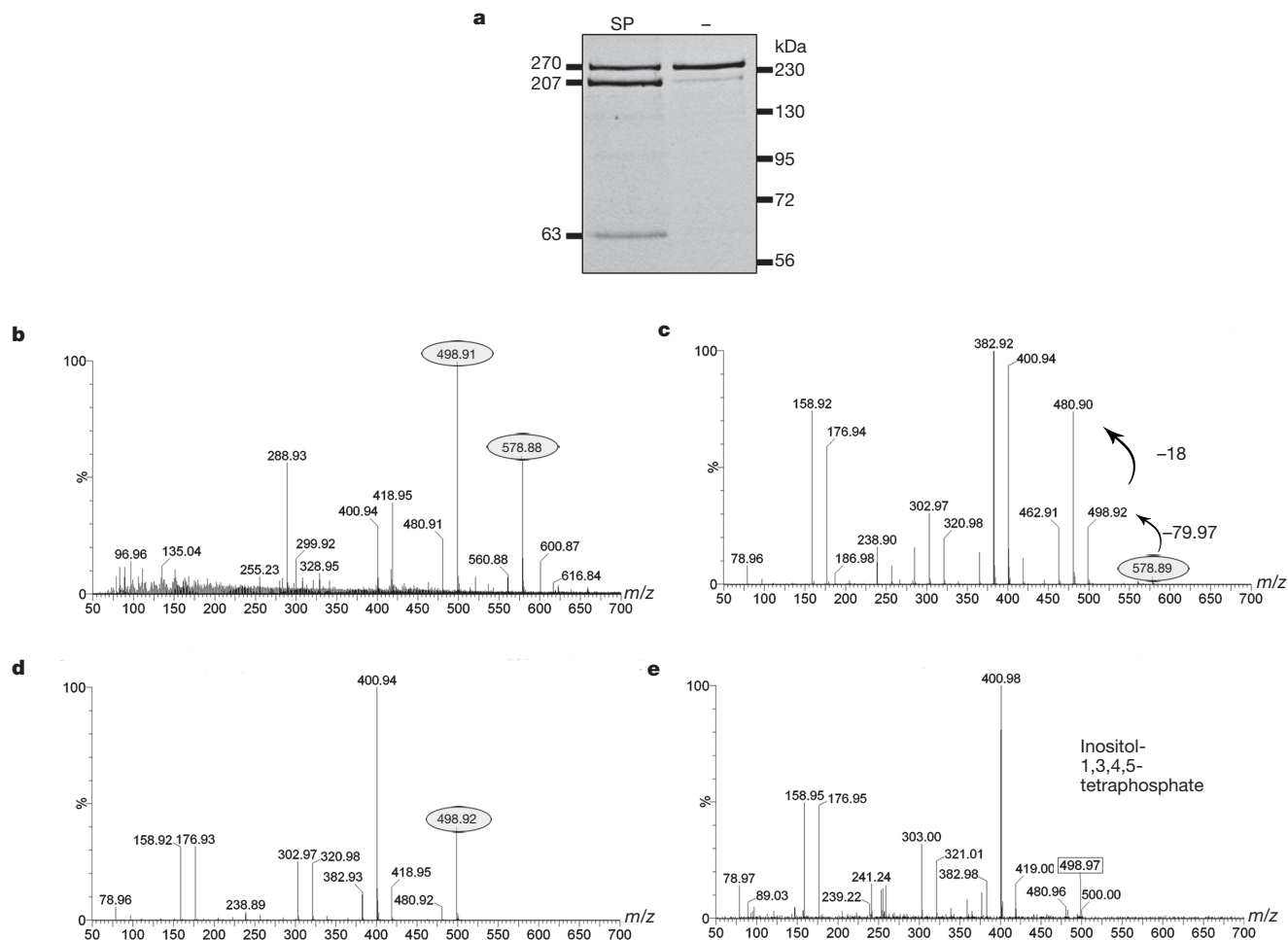


Figure 1 | Purified cytosolic splenocyte extract in the *in vitro* TcdB cleavage assay and mass spectrometry. **a**, *In vitro* cleavage reaction. Cleavage of fluorescently labelled Cy3-TcdB activated by splenocyte cytosolic extract (SP) and the same reaction without SP (–); visualization was by Cy3 fluorescence. **b–e**, Active fractions of the splenocyte extract purification (Supplementary Fig. 1) were analysed by mass spectrometry; representative spectra are shown. InsP_x-peaks from the purified porcine splenocyte extract are encircled. The y axis measures MS-intensity as a per cent of the most

intense peak. *m/z*, mass-to-charge ratio. **b**, Survey spectra from fraction C5 of S75 size exclusion chromatography (Supplementary Fig. 1f) were acquired over a mass range from 50–1,500 Da. **c**, **d**, From fraction C5 the peaks at 578.88/578.89 Da (encircled and shaded) and 498.91/498.92 Da (encircled and shaded) were manually selected for fragmentation in MS/MS mode (shown in **c** and **d**, respectively). **e**, The MS/MS fragment spectrum of commercially available inositol-1,3,4,5-tetraphosphate (boxed, with a peak at 498.97) served as a positive control.

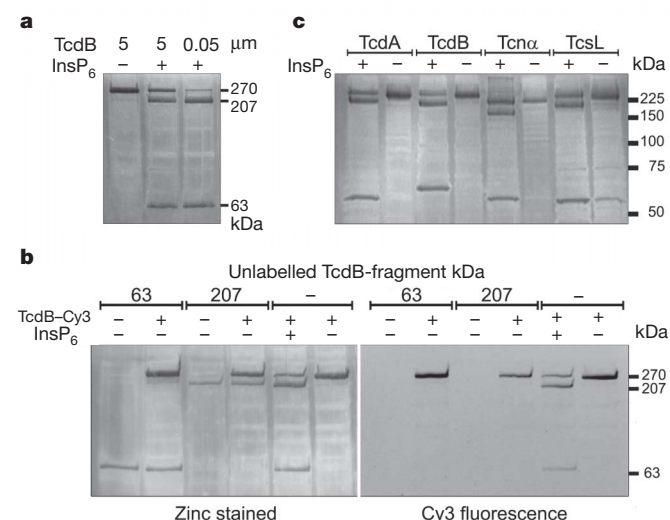


Figure 2 | In vitro cleavage of LCTs by purified splenocyte extracts or inositolhexaphosphate. **a**, Cleavage of native TcdB induced by the addition of InsP₆. Unlabelled toxin (16 μ g) was incubated with InsP₆ (at 100 μ M concentration) at different TcdB concentrations (5.0 or 0.05 μ M); following cleavage, the solutions were lyophilized, resuspended in water (approximately 1 μ g) subjected to SDS-PAGE and detected with zinc staining. **b**, Exclusion of a cascade reaction. Cy3-labelled TcdB was co-incubated with the isolated (pre-activated) 63 kDa catalytic-DXD or the 207 kDa ligand-translocation domain, respectively. Left panel, zinc staining; right panel, the same gel visualized by Cy3 fluorescence. **c**, Cleavage of LCTs from different *Clostridia*. Assay without (–) or with (+) activation by 10 mM InsP₆. LCTs were: TcdA and TcdB of *C. difficile* strain VPI10463; Tcn α of *C. novyi* strain 19402; and TcsL of *C. sordellii* strain IP-82. Visualization was by zinc staining.

active centre of the protease. EPNP (1,2-epoxy-3-(*p*-nitrophenoxy)propane) is a known inhibitor of aspartate proteases, which, in contrast to pepstatin A, covalently binds to the active site³⁹. To determine the TcdB intrinsic protease active site we thus generated an ‘EPNP-derivative’ of TcdB. Following addition of InsP₆, this modified TcdB was no longer cleaved (data not shown). This suggests that TcdB had lost its aspartate protease function. The active site of aspartate proteases is known to be situated in the motif DXG (where X is S or T)³⁹. According to database searches (see Supplementary Information), TcdB contains three of these motifs at positions 1665, 2001 and 2133. To exclude the possibility that EPNP modified a contaminating protease rather than TcdB, and to identify its active site, we analysed fragments generated by tryptic proteolysis of EPNP-treated and untreated TcdB by nanoESI-QTOF mass spectrometry. Only a single fragment with an EPNP-induced mass shift of 195 Da was detected. The triple charged fragment had an apparent mass of $m/z = 1075.49$ and was confirmed by MS/MS analysis to be amino acids 1652–1678 of TcdB (Supplementary Fig. 4). This fragment contains the aspartate protease consensus motif DSG at amino acid position 1665, confirming the predicted DSG(1665) to be the active site of the autocatalytic TcdB protease. Furthermore, EPNP pretreatment completely abrogated cellular toxicity when the toxin was added to the target cell culture (Fig. 3c). As expected, loss of cytotoxicity was not due to modification of the catalytic-DXD glucosyltransferase domain, because microinjection of EPNP-treated TcdB induced cell rounding (Fig. 3f).

To dissect further the autocatalytic cleavage mechanism, we isolated both N- and C-terminal fragments by anion exchange chromatography and tested their proteolytic activity. As indicated in Fig. 2b, neither the 63 kDa nor the 207 kDa fragment was able to cleave full-length Cy3-labelled TcdB in the absence of InsP₆.

Thus the cleavage reaction of TcdB is intramolecular and not followed by a cascade reaction catalysed by its cleavage fragments.

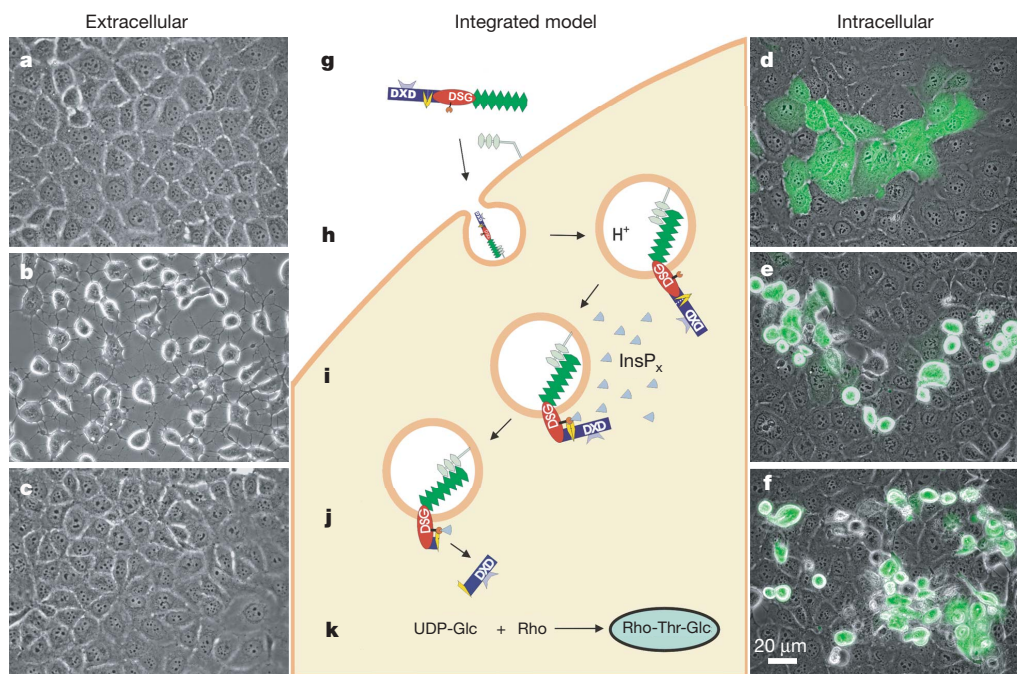


Figure 3 | An integrated model of events necessary for the cellular cytotoxic action of TcdB. **a–c**, Cytotoxic effects of TcdB (1 μ g ml^{–1}) applied to the supernatant of IHKE cells. **a**, Control buffer without TcdB. **b**, Unmodified TcdB. **c**, EPNP-modified TcdB. **d–f**, Effects of TcdB microinjected into the cytosol of IHKE cells. **d**, Control buffer (containing dextran-FITC) without TcdB. **e**, Unmodified TcdB. **f**, EPNP-modified TcdB. **g–k**, Schematic model of TcdB uptake and delivery of the catalytic-DXD domain into the eukaryotic cell. Colour code: dark green, the TcdB ligand domain; red, the TcdB translocation domain; dark blue, the TcdB catalytic-DXD domain; light grey-green, the

receptor for TcdB; brown, cellular membranes; and light blue, cytosolic inositolphosphates. **g**, Interaction with cell receptors induces receptor-mediated endocytosis. **h**, The acidic pH of the endosome triggers the first conformational change and results in pore formation of the ligand-translocation domain. **i**, Cytosolic InsP₆ interacts with TcdB and induces a second conformational change, activating the intrinsic protease function. **j**, Autocatalytic cleavage and release of the catalytic-DXD glucosyltransferase domain into the cytosol. **k**, Glucosylation of the cytosolic target GTPases, leading to disaggregation of the cytoskeleton and cell death.

Furthermore, Fig. 2b shows that TcdB loses its intrinsic protease function following cleavage.

Autocatalytic cleavage of other LCTs. Assuming autocatalytic activation, we expected that other members of the LCT family^{12,40} would show similar properties. An assay with InsP₆-activated *C. sordellii* TcsL, *C. novyi* Tcn α and *C. difficile* TcdA demonstrated cleavage like that observed with TcdB (Fig. 2c). Apparently, InsP_x-induced autocatalytic cleavage is a common property of all LCTs.

Model for the uptake and activation of toxin B. A model of the events occurring within the cells is presented in Fig. 3. On binding to host cell receptors^{22,23} (Fig. 3g) the toxins are endocytosed²⁴. The low pH in endosomes triggers the first conformational change of the toxin leading to membrane insertion^{26,28,41} (Fig. 3h), followed by translocation of the N-terminal catalytic-DXD domain into the cytosol^{20,29}.

The translocation-ligand domain remains associated with endosomal membranes and only the catalytic-DXD domain penetrates into the cytosol^{20,29}. We show here that cytosolic InsP_x is needed to induce autocatalytic cleavage of TcdB. It is known that in eukaryotic cells InsPs may induce conformational changes of proteins^{42,43}, and therefore we postulate that TcdB undergoes a second conformational change (Fig. 3i), which is induced by InsP_x and immediately leads to autocatalytic proteolysis and delivery of the catalytic-DXD domain into the cytosol (Fig. 3j). Because our experimental data show that the intramolecular reaction quickly follows the addition of InsP₆, cleavage of the glucosyltransferase portion could immediately occur on its translocation into the cytosol. Additionally, our data using EPNP-modified TcdB show that InsP_x-induced autocatalytic cleavage is absolutely required for the execution of TcdB's biological function. Once the toxin's aspartate protease function is blocked (Fig. 3c), the catalytic-DXD domain can no longer be cleaved off and thus, according to other researchers^{20,29}, the DXD domain can no longer reach the cytosol. Following its translocation and release, the catalytic-DXD fragment acts on its cytosolic targets (Fig. 3k), the GTPases of the Rho/Rac family, leading to the observed blockade of signal transduction processes and, consequently, the disaggregation of the cytoskeleton and cell death^{12,31,40}.

Discussion

In conclusion, we identified InsP_x as the host factor required for the autocatalytic proteolysis of TcdB. Cellular inositolphosphates are involved in the regulation of a large variety of cellular processes in the cytoplasm, the plasma membrane and the nucleus^{43,44}. Recently, it was reported that InsP₆ is required for the correct folding of ADAR2, involved in RNA editing⁴². However, InsP_x as a cofactor for activation of proteolytic activity has not been reported before, to the best of our knowledge. Furthermore, our data and some recent reports^{42,43} indicate that in eukaryotic cells InsPs serve more generally as cofactors for proper protein folding and function. The proof of InsP₆-induced cleavage of LCTs shown in Fig. 2c leads us to propose InsP_x-inducible proteolytic cleavage as an additional feature common to the LCT family. However, in contrast to TcdA, TcdB and TcsL, which have a DXG-motif at the same position, Tcn α has no DXG-site and therefore should possess a different protease function to the aspartate protease of the other LCTs.

Regarding the similar genomic organization of LCTs⁴⁵, it is not surprising that they carry their intramolecular protease with them because this combines complete mechanisms of regulated production, release of toxins and induced self-activation at the final site of action⁴⁶. During evolution, LCT-producing *Clostridia* have thus gained a very effective means of controlling the energy-demanding use of their toxins.

METHODS

Labelling of toxins with Cy3. The toxins TcdB and TcdA produced by the toxin high-producer strain VPI10463 were purchased from tgcBIOMICS (Mainz,

Germany). Aliquots of 200–400 μ g were transferred to NaHCO₃ buffer by gel filtration and labelled with Cy3 (GE Healthcare) according to the manufacturer's instructions. Unbound dye was removed by size exclusion chromatography using 10 mM Tris-HCl, pH 8.5, as running buffer. The molar dye/toxin-labelling ratio amounted to 0.8–1.6. Labelled toxins were aliquoted and stored at -80°C .

In vitro cleavage assay. The cleavage assay was performed as described previously²⁹, in Tris-HCl, pH 8.5, buffer at a volume of 10 μ l (unless stated otherwise) consisting of 500 ng of labelled toxin or 1 μ g of unlabelled toxin, 3 μ l of cell extract and purified fractions or commercially available inositol phosphates (purchased from Axxora, Merck, and Sigma, respectively). Following a 30 min incubation at 25°C , samples were separated by SDS-PAGE (10%), Cy3-labelled toxin and toxin fragments were visualized by fluorescence, unlabelled toxins were visualized by zinc staining and both were documented with a Gel Doc EQ System Image Reader (Bio-Rad).

Preparation of cytosolic porcine splenocyte extract. Freshly isolated pig spleens were prepared in phosphate buffered saline (PBS) and processed into single-cell suspensions. Erythrocytes were lysed by adding low-salt buffer (150 mM NH₄Cl, 1 mM KHCO₃ and 0.1 mM EDTA, pH 7.6). Splenocytes were washed twice with 10 mM Tris-HCl, pH 8.5, and the pellet was immediately frozen at -80°C . Cell extracts were obtained by diluting the splenocyte pellet 1:1 with 10 mM Tris-HCl, pH 8.5, followed by mechanical lysis in a Potter-Elvehjem homogenizer (20 strokes, 1,000 r.p.m.). The lysate was centrifuged for 1 h at $200,000 \times g$ at 4°C and the supernatant was promptly used for further experiments.

Purification of active fractions from porcine splenocyte extract. Details of purification are given in Supplementary Fig. 1.

Mass spectrometry. Lyophilized active fractions were dissolved in 50% acetonitrile/water (liquid-chromatography-MS grade, Merck). Samples (1–2 μ l) were applied to a gold-coated glass capillary (Type C nanoflow needle, Waters) and introduced into the nano-ESI source using a capillary voltage of 500–800 V, resulting in a spray of about 50 nl min⁻¹.

Mass detection was performed on a hybrid orthogonal acceleration QTOF MS/MS (Q-TOF Premier, Waters) equipped with an ESI interface and operated in a negative ionization mode. The mass spectrometer was operated in V-mode and tuned to a resolution of 10,000 full-width at half-maximum (FWHM) for singly charged ions. Prominent peaks in the survey mass spectrometry spectrum were manually selected for fragmentation in MS/MS mode using collision energies between 15 and 30 eV.

Affinity purification of TcdB. TcdB-specific monoclonal antibody 2CV (7 mg)³⁷ was coupled to a HiTrap NHS Sepharose column (GE Healthcare), according to the manufacturer's protocol. Approximately 4 mg of conventionally purified TcdB were applied to this column and washed with a buffer containing 50 mM Tris/HCl, pH 7.0 and 125 mM NaCl. TcdB was eluted with 0.1 M triethanolamine-HCl, pH 11, and immediately neutralized with a one-tenth volume of 1 M Tris-HCl, pH 7.5.

EPNP modification of TcdB. TcdB was transferred to a buffer containing 250 mM sodium acetate, 500 mM NaCl, 1 mM EDTA, 500 μ M DTT and 5% DMSO (pH 5.5). EPNP (10 mM; Acros Organics, Belgium) was added and incubated for 30 h at 500 r.p.m. on an orbital mixer at room temperature. Finally the buffer was changed (ZebaSpin desalting columns, Pierce) to HEPES 40 mM, pH 7.5.

Microinjection experiments. Microinjection experiments were performed with IHKE cells maintained in DMEM/HAM's F12-medium (1:1) supplemented with 10% FCS and 2 mM glutamine in the presence of 65 mg l⁻¹ penicillin-G and 100 mg l⁻¹ streptomycin. IHKE cells were seeded overnight on 35 mm Petri dishes with glass bottoms and then used in the experiments. Native or EPNP-modified TcdB were diluted in an intracellular buffer (145 mM potassium glutamate, 20 mM NaCl, pH 7.3) to a final concentration of 1 μ g ml⁻¹. The intracellular buffer contained fluorescein isothiocyanate (FITC)-labelled dextran (100 mg ml⁻¹, molecular mass approximately 19,500 Da; Sigma) to visualize successful microinjection. Microinjection was performed with an Eppendorf micromanipulator 5170 and microinjector 5242 system (Eppendorf AG). Injection pulses of 120 hPa were applied for 0.3 s. Images were taken at several time points (2 h, 7 h and 24 h) and acquired with an inverted Leitz DM-IRB phase-contrast microscope (Leica Microsystems) equipped with a VisiTron 'VisiChrome' High Speed Fluorescence Polychromatorsystem (VisiTron Systems). Excitation and emission wavelengths for fluorescence images were 490 and 525 nm.

For data acquisition, storage and processing we used Digital Camera Photometrics 'Coolsnap cf' (Roper Scientific) and Metafluor acquisition software (Universal Imaging Corporation).

Mass spectrometry of native and EPNP-modified TcdB. Details of the mass spectrometry are given in Supplementary Fig. 4.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Special Feature

Vaccine adjuvants: Current state and future trends

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Summary The problem with pure recombinant or synthetic antigens used in modern day vaccines is that they are generally far less immunogenic than older style live or killed whole organism vaccines. This has created a major need for improved and more powerful adjuvants for use in these vaccines. With few exceptions, alum remains the sole adjuvant approved for human use in the majority of countries worldwide. Although alum is able to induce a good antibody (Th2) response, it has little capacity to stimulate cellular (Th1) immune responses which are so important for protection against many pathogens. In addition, alum has the potential to cause severe local and systemic side-effects including sterile abscesses, eosinophilia and myofascitis, although fortunately most of the more serious side-effects are relatively rare. There is also community concern regarding the possible role of aluminium in neurodegenerative diseases such as Alzheimer's disease. Consequently, there is a major unmet need for safer and more effective adjuvants suitable for human use. In particular, there is demand for safe and non-toxic adjuvants able to stimulate cellular (Th1) immunity. Other needs in light of new vaccine technologies are adjuvants suitable for use with mucosally-delivered vaccines, DNA vaccines, cancer and autoimmunity vaccines. Each of these areas are highly specialized with their own unique needs in respect of suitable adjuvant technology. This paper reviews the state of the art in the adjuvant field, explores future directions of adjuvant development and finally examines some of the impediments and barriers to development and registration of new human adjuvants.

Key words: adjuvants, immune response, mucosal immunity, vaccines.

Adjuvant origins

The goal of vaccination is the generation of a strong immune response to the administered antigen able to provide long-term protection against infection. To achieve this objective with killed as opposed to live attenuated vaccines, often requires the addition of an adjuvant.¹ Adjuvants are compounds that enhance the specific immune response against co-inoculated antigens. The word adjuvant comes from the Latin word *adjuvare*, which means to help or to enhance.² The concept of adjuvants arose in the 1920s from observations such as those of Ramon *et al.* who noted that horses that developed an abscess at the inoculation site of diphtheria toxoid generated higher specific antibody titres.^{3,4} They subsequently found that an abscess generated by the injection of unrelated substances along with the diphtheria toxoid increased the immune response against the toxoid.^{3,4} The adjuvant activity of aluminium compounds was demonstrated by Glenny *et al.* in 1926 with diphtheria toxoid absorbed to alum.⁵ To this day, aluminium-based compounds (principally aluminium phosphate or hydroxide) remain the predominant human adjuvants.⁶ In 1936, Freund developed an emulsion of water and mineral oil containing killed mycobacteria, thereby creating one of the most potent known adjuvants, Freund's complete adjuvant (FCA).^{7,8} Despite being the gold standard adjuvant, FCA causes severe local reactions and is considered

too toxic for human use. The oil in water emulsion without added mycobacteria is known as Freund's incomplete adjuvant (FIA) and, being less toxic, has been used in human vaccine formulations.⁸ In the 1950s, Johnson *et al.* found that lipopolysaccharides (LPS) from Gram-negative bacteria exhibited adjuvant activity⁹ and detoxified LPS or related compounds such as lipid A have since been used as adjuvants in human studies.¹⁰ In 1974, Lederer *et al.* identified muramyl dipeptide (MDP) as a mycobacterial component with adjuvant activity contained in FCA.¹¹ Bacterial components are often potent immune activators although commonly associated with toxicity, for example, bacterial DNA with immunostimulatory CpG motifs is one of the most potent cellular adjuvants.¹² Immunostimulatory CpG are unmethylated cytosine-guanine dinucleotides found in bacterial DNA but absent in mammalian DNA. Overall, several hundred natural and synthetic compounds have been identified to have adjuvant activity. Although a significant number are clearly more potent than alum, toxicity is perhaps the single most important impediment in introducing most such adjuvants to human use.²

Adjuvant roles

Adjuvants can be used for various purposes: (i) to enhance the immunogenicity of highly purified or recombinant antigens; (ii) to reduce the amount of antigen or the number of immunizations needed for protective immunity; (iii) to improve the efficacy of vaccines in newborns, the elderly or immunocompromised persons; or (iv) as antigen delivery systems for the uptake of antigens by the mucosa.^{13–15}

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Adjuvant selection

Some of the features involved in adjuvant selection are: the antigen, the species to be vaccinated, the route of administration and the likelihood of side-effects.^{16,17} Ideally, adjuvants should be stable with long shelf life, biodegradable, cheap to produce, not induce immune responses against themselves and promote an appropriate immune response (i.e. cellular or antibody immunity depending on requirements for protection).¹⁸ There are marked differences in the efficacy of adjuvants depending on the administration route (e.g. between mucosal and parenteral routes). Hence new vectors, antigen delivery systems or adjuvant compounds need to take into account the characteristics of the proposed administration route.¹⁹

Adjuvant safety issues

The benefits flowing from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions.^{20,21} Adverse reactions to adjuvants can be classified as local or systemic. Important local reactions include pain, local inflammation, swelling, injection site necrosis, lymphadenopathy, granulomas, ulcers and the generation of sterile abscesses. Systemic reactions include nausea, fever, adjuvant arthritis, uveitis, eosinophilia, allergy, anaphylaxis, organ specific toxicity and immunotoxicity (i.e. the liberation of cytokines, immunosuppression or autoimmune diseases).^{22,23} Unfortunately, potent adjuvant action is often correlated with increased toxicity, as exemplified by the case of FCA which although potent is too toxic for human use. Thus, one of the major challenges in adjuvant research is to gain potency while minimizing toxicity. The difficulty of achieving this objective is reflected in the fact that alum, despite being initially discovered over 80 years ago, remains the dominant human adjuvant in use today.

Adjuvant regulatory requirements

Regulations for the human use of adjuvants are far more rigorous than those applied to veterinary vaccines. In addition to preclinical studies on the adjuvant itself, the combined antigen-adjuvant formulation also needs to be subjected to toxicology prior to commencement of phase 1 clinical trials.²⁴ The toxicological evaluation is normally conducted in small animal species such as mice, rats or rabbits and should use the same administration route proposed for human use. The dose and frequency of vaccination for preclinical toxicology should be similar to or higher than the proposed human dose to maximize the ability to identify potential safety problems.²⁵ Preclinical studies may also help in selecting the optimum vaccine dose.²⁶

Adjuvant classification

Adjuvants can be classified according to their source, mechanism of action or physicochemical properties.² Edelman²² classified adjuvants into three groups: (i) active immunostimulants, being substances that increase the immune response to the antigen; (ii) carriers, being immunogenic proteins that provide T-cell help; and (iii) vehicle adjuvants, being oil

emulsions or liposomes that serve as a matrix for antigens as well as stimulating the immune response. An alternative adjuvant classification divides adjuvants according to administration route, namely mucosal or parenteral. A third classification divides adjuvants into alum salts and other mineral adjuvants; tensoactive agents; bacterial derivatives; vehicles and slow release materials or cytokines.¹⁷ A fourth more recently proposed system of classification divides adjuvants into the following groups: gel-based adjuvants, tensoactive agents, bacterial products, oil emulsions, particulated adjuvants, fusion proteins or lipopeptides.²⁷

Adjuvant limitations

In spite of progress in the identification of mechanisms of adjuvant action, alum remains the dominant adjuvant for human vaccines. Although many other adjuvants have been proposed over the years, these have failed to be successful in humans largely because of toxicity, stability, bioavailability and cost. Because of effects of size, electric charge and hydrophobicity which regulate the incorporation of proteins into the adjuvant formulation, it is difficult to predict on an empirical basis which adjuvant will work most effectively with a particular protein or peptide. Moreover, epitope modifications may occur during formulation or conjugation. In the case of carrier proteins, a pre-existing immunity to the carrier protein is a major limitation.²⁷ Furthermore, each adjuvant generates a characteristic immune response profile. For example, the inability of alum-based adjuvants to induce Th1 antibody isotypes or cellular immune responses, and their poor adjuvant effect on polysaccharide antigens limit their applicability to many vaccines.¹⁶

Major adjuvant groups

Mineral salt adjuvants

Alum-based adjuvants

Since the experiments of Glenney *et al.*⁵ alum salts, principally aluminium phosphate or hydroxide, have been the most widely used human adjuvants.¹⁶ Unfortunately, alum salts are relatively weak adjuvants and rarely induce cellular immune responses.^{28–30} Studies suggest alum salts work by causing the formation of an antigen depot at the inoculation site from where antigen is released slowly.³¹ The trapping of soluble antigen in the alum gel may also increase the duration of antigen interaction with the immune system. Other mechanisms of action involve complement, eosinophil and macrophage, activation³² and increased efficiency of antigen uptake by antigen presenting cells seen with particulate matter with a size under 10 μm .³³

Whilst alum-based vaccines are generally well tolerated, granulomas are common when the subcutaneous or intradermal route is used rather than intramuscular injection.^{34,35} Other specific limitations of alum adjuvants are increased IgE production, allergenicity^{30,33,34,36–38} and neurotoxicity. Although under normal circumstances low doses of aluminium are excreted by the kidneys, under certain conditions such as reduced renal function, aluminium is accumulated in the body and becomes highly toxic. High aluminium levels in the body predominately affect the brain and bone tissues causing

fatal neurological syndrome and dialysis-associated dementia. Aluminium intoxication has also been associated with amyotrophic lateral sclerosis and Alzheimer's disease.

Other mineral salt adjuvants

The salts of calcium, iron and zirconium have also been used to adsorb antigens, although not to the extent of alum salts.³⁴ In particular, calcium phosphate has been used for diphtheria-tetanus-pertussis vaccines.^{39,40} While having similar properties to alum salts, calcium phosphate has the advantage that it is a natural compound to the human body and is therefore exceptionally well tolerated. It has a reasonable capacity to adsorb antigens, induces high levels of IgG antibodies and does not increase IgE production. Neurological reactions to pertussis vaccines adsorbed to calcium phosphate are rare.⁴¹

Tensoactive adjuvants

Quil A is a saponin derived from an aqueous extract from the bark of *Quillaja saponaria*. Fractions purified from this extract by reverse phase chromatography, mainly QS-21, have been studied as alternatives to alum when strong cellular responses are required for a particular vaccine.^{22,42,43} Saponins are tensoactive glycosides containing a hydrophobic nucleus of triterpenoid structure with carbohydrate chains linked to the nucleus.⁴² Saponins induce a strong adjuvant effect to T-dependent as well as T-independent antigens. Saponins also induce strong cytotoxic CD8⁺ lymphocyte responses and potentiate the response to mucosal antigens.⁴² Quil A has been used successfully for veterinary applications.⁴⁴ It is a natural product composed of more than 23 different saponins and is generally considered too toxic for human use. In addition to severe local reactions and granulomas, toxicity includes severe haemolysis reflecting the affinity of saponins for cholesterol present in erythrocyte membranes, resulting in membrane solubilization and haemolysis.^{20,44–47} The Quil A-derived saponin QS-21, whilst less toxic than Quil A itself, has many of the same problems and may similarly prove unsuitable for most human uses other than therapeutic vaccines for life threatening illnesses such as HIV infection.⁴⁸

Bacteria-derived adjuvants

Given their potent immunostimulatory capacity, bacteria-derived substances constitute a major potential source of adjuvants. Cell wall peptidoglycan or lipopolysaccharide of Gram-negative bacteria, enhance the immune response against co-administered antigens despite themselves not being very immunogenic. This adjuvant activity is mediated through activation of Toll-like receptors that mediate the danger signals activating the host immune defence system.³⁶ Different species of bacteria used as a source of adjuvants include *Mycobacterium* spp., *Corynebacterium parvum*, *C. granulatum*, *Bordetella pertussis* and *Neisseria meningitidis*. Unfortunately, as whole alive or killed microorganisms these are too toxic to be used as human adjuvants.²³ However, much of the adjuvant activity of these bacteria is mediated by N-acetyl muramyl-L-alanyl-D-isoglutamine, also called MDP. The adjuvant activity of MDP depends on the administration conditions.^{11,49} In saline, it mainly enhances humoral immunity^{20,50,51} whilst when incorporated into liposomes or

mixed with glycerol it induces strong cellular immunity.⁵² MDP activates many different cell types including macrophages, leucocytes, mastocytes, endothelial cells and fibroblasts inducing the secretion of cytokines such as IL-1, B-cell growth factor and fibroblast activating factor. MDP also induces an increase in the production of superoxides, prostaglandins and collagenase.⁵³ Different compounds derived from MDP include threonyl-MDP, a potent yet non-pyrogenic adjuvant.²³

Another important group of compounds derived from the cell wall of Gram-negative bacteria are the lipopolysaccharides (LPS). They are potent B-cell mitogens, but also activate T cells to produce IFN- γ and TNF and thereby enhance cellular immune responses. The major structural element responsible for their toxicity and adjuvant effect is Lipid A. In low acid conditions, lipid A can be hydrolysed to obtain monophosphoryl lipid A, a compound which retains the adjuvant activity of Lipid A with reduced toxicity.⁵⁴ Another extract from bacterial walls is trehalose dimycolate (TDM), an adjuvant which simulates both humoral and cellular responses.⁵⁵ The demonstration that mycobacterial DNA had adjuvant activity, led to the discovery that the adjuvant activity correlated with a higher content of CpG motifs present in bacterial nucleic acids. DNA containing CpG motifs is one of the most potent cellular adjuvants.¹²

Adjuvant emulsions

This class includes oil in water or water in oil emulsions such as FIA, Montanide, Adjuvant 65, and Lipovant.¹⁷ The mechanism of action of adjuvant emulsions includes the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of antibody producing plasma cells.⁵⁶ In general, these adjuvants are too toxic for routine human prophylactic vaccine use, although they may be suitable for use in terminal conditions such as cancer where there is a greater tolerance of side-effects. Frequent side-effects of emulsions include inflammatory reactions, granulomas and ulcers at the injection site. Various types of emulsions have been used, with different natural oils, in order to find more stable, potent and less toxic formulations. Adjuvant 65 offers the advantage over the mineral oil used in IFA^{57–59} that it can be metabolized.²³ Different emulsions like oil in water⁶⁰ and water in oil in water⁶¹ have been developed with the latter being as potent as IFA but more stable, less viscous and easier to administer with less resulting granulomas.^{62,63} Montanide is a family of oil-based adjuvants that have been used in experimental vaccines in mice, rats, cats and dogs, using natural, recombinant and synthetic antigens. In humans, Montanide has been used in trial vaccines against HIV, malaria and breast cancer.⁶⁴

Liposome adjuvants

Liposomes are synthetic spheres consisting of lipid layers that can encapsulate antigens and act as both a vaccine delivery vehicle and adjuvant. Liposomes have been used widely in experimental vaccines. The potency of liposomes depends on the number of lipid layers,⁶⁵ electric charge,⁶⁶ composition⁶⁷ and method of preparation.^{67–69} They enhance both humoral and cellular immunity to protein and polysaccharide antigens.^{66,69}

Liposomes help extend the half-life of antigens in blood ensuring a higher antigen exposure to antigen presenting cells after vaccination.⁷⁰ Stability, manufacturing and quality assurance problems seem to have been major factors behind the fact that as yet no adjuvant based on liposomes has been registered for human use.

Polymeric microsphere adjuvants

Among particulated and polymeric systems, poly (DL-lactide-coglycolide) microspheres have been extensively studied. These are biocompatible and biodegradable microspheres able to incorporate different antigens. One of the advantages of this system is the capacity to manipulate the degradation kinetics of the microspheres by varying the relative concentration of their components, thereby controlling the time of antigen release.^{71,72}

Cytokines as adjuvants

Cytokines are included in the modern classification of adjuvants. IFN- γ is a pleiotropic cytokine able to enhance cellular immune responses through a variety of mechanisms.⁷³ Granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the primary immune response by activating and recruiting antigen presenting cells.⁷⁴ The practical application of GM-CSF as an adjuvant has been limited by the requirement for multiple doses, toxicity and the immunogenicity of heterologous cytokines.¹⁷ Cytokines are particularly seen to have potential in DNA vaccines where the cytokine can be expressed by the same vector as the antigen.

Carbohydrate adjuvants

Inulin-derived adjuvants

Several complex carbohydrates of natural origin stimulate cells from the immune and reticulo-endothelial system.⁷⁵ The main source of these polysaccharides have been plants and fungus. Gamma inulin, a carbohydrate derived from plant roots of the Compositae family, is a potent humoral and cellular immune adjuvant. Gamma inulin is a potent alternate complement pathway activator increasing production of activated C3 and thereby activating macrophages.⁷⁶ Gamma inulin is effective at boosting cellular immune responses without the toxicity exhibited by other adjuvants such as FCA. Gamma inulin can be combined with a variety of other adjuvant components, for example, aluminium hydroxide, to produce a range of tailor made adjuvants with varying degrees of Th1 and Th2 activity. For example, Algammulin is a combination of γ -inulin and aluminium hydroxide. Algammulin exhibits a higher ratio of Th2 to Th1 activity than γ -inulin alone, its overall effect being equivalent to alum despite having a lower overall alum content.^{77,78} Inulin-based adjuvants have successfully been tested in multiple animal models in combination with such antigens as diphtheria and tetanus toxoid, respiratory syncytial virus, the E7 protein from the Human Papilloma Virus, Herpes Virus 2 glycoprotein D, Hepatitis B surface antigen, influenza haemagglutinin, Haemophilus influenzae antigens and antigens from *Plasmodium falciparum*. Major advantages of inulin-derived

adjuvants are that they induce both Th1 and Th2 immune responses, unlike alum do not induce IgE, and are not associated with any significant local or systemic toxicity.⁷⁹ Inulin is metabolisable into simple sugars fructose and glucose. Inulin does not, therefore, suffer from concerns regarding long-term accumulation and toxicity that are associated with metal-based compounds such as alum.

Other carbohydrate adjuvants

Polysaccharides based on glucose and mannose which have adjuvant action include glucans, dextrans, lentinans, glucomannans and galactomannans. Levans and xylans,⁸⁰ (82) also have immuno-enhancing activity. Macrophages have glucan and mannan receptors, activation of which stimulates phagocytosis and cytokine secretion plus release of leukotrienes and prostaglandins. Polysaccharides have been used for immune stimulation in patients with cancer.⁸¹ *In vitro*, mannan activates monocytes and macrophages to secrete IFN, TNF, GM-CSF, IL-1 and IL-6.⁸² Acemannan, a natural polysaccharide extracted as a mucilaginous gel of the *Aloe barbadensis*, stimulates generation of cytotoxic T lymphocytes (CTL)⁸³ and the cytotoxic activity of NK cells.⁸⁴ Recently, acemannan has been shown to enhance the immune response to nasally administered Hepatitis B surface antigen (HBsAg), generating similar levels of IgG antibody titres in sera compared to the immune response generated by an intramuscular alum-based HBsAg control vaccine.⁸⁵

Adjuvant formulations

New adjuvant formulations have resulted from the mixture of different adjuvants in the same formulation. As a general rule, two or more adjuvants with different mechanisms of action are combined to enhance the potency and type of the immune response to the vaccine antigen. For example, alum salts can be formulated in combination with other adjuvants such as Lipid A to increase immunogenicity. Similarly algammulin which is the combination of γ -inulin plus alum has increased absorptive capacity and increased ability to stimulate Th2 responses.⁷⁷ Saponins such as Quil A have also been used as a part of immunostimulatory complexes (ISCOMS).⁴² ISCOMS are virus like particles of 30–40 nm and dodecahedral structure, composed by Quil A, lipids and cholesterol. Antigens can be inserted in the membrane or encapsulated. A wide variety of proteins have been inserted in these cage-like structures.^{86–88} ISCOMS can be used through the oral, respiratory and vaginal routes.⁸⁹ ISCOMS are particularly effective in activating cellular immunity and cytotoxic T cells⁴² but often have problems with stability and toxicity.

Mucosal adjuvants

The development of adjuvants for mucosal immunization is an important current area of vaccine development. The quality of mucosal adjuvants needs to take into account the ability to stimulate the uptake of antigen through the various mucosal routes, and its ability to enhance the immunogenicity of mucosally-delivered antigen. Different results can be obtained for the same adjuvant when administered by a parenteral or mucosal route. Alum salts, the most widely used parenteral adjuvants, are ineffective when administered by the oral or

nasal route.⁹⁰ The mucosa is a door of entry for many pathogens. Although it is very difficult to generate mucosal antibodies through parenteral vaccination, it is possible to obtain mucosal as well as parenteral immunity by inoculating antigen by the mucosal route.⁹¹ For pathogens colonizing mucosal surfaces or those having a mucosal route of entry, protection correlates well with a strong local mucosal response.⁹² For mucosal immunization, several adjuvant strategies involve binding or coating with specific ligands to deliver the antigens to specialized epithelial cells (M cells). It is also important to correctly match the physicochemical characteristics of the antigen-like size, electric charge, and hydrophobicity to let the antigen cross mucosal barriers.¹⁹ After optimization of these characteristics, the selected adjuvant may also enhance the immune response by mechanisms already described: adsorption and depot effect, cytokine induction, complement activation, recruiting of different cell populations, the delivery to different APC, the regulation of the expression via MHC class I or class II and the stimulation of the production of different subtypes of antibodies.⁹³

Bacterial derivatives

Some well known parenteral adjuvants, like MDP, monophosphoryl lipid A (MPL) and LPS, also act as mucosal adjuvants. Compounds like bacterial toxins of *Vibrio cholerae* (CT) and *Escherichia coli* (HLT) and their respective toxoids are particularly useful mucosal adjuvants.^{94–96} Although CT remains one of the most potent known mucosal adjuvants, it suffers from high toxicity and also induces a strong immune response against itself. The strong adjuvanticity of CT and HLT may be explained by their ability to increase antigen presentation by B cell, B-cell differentiation to IgA secreting cells, interaction with T cells and increase cytokine production.⁹⁷ The B subunit of CT is less toxic and strategies have been taken to mutate the gene coding for CT in order to detoxify the cholera toxin.

Synthetic or inactivated antigen delivery systems

This group of mucosal adjuvants includes different synthetic polymeric particles composed by biodegradable poly(DL-lactide-co-glycolide) (DL-PLG), cellulose acetate, iminocarbonates, proteinoid microspheres, polyanhydrides, dextrans, as well as particles produced from natural materials like alginates, gelatine and plant seeds. Other natural compounds like liposomes, virosomes and ISCOMS can also be included in this group.⁹⁸ Particle size is one of the major factors involved in the mucosal delivery. It has been shown that particles over 10 µm are not adsorbed by the intestinal mucosa.⁹⁹ Lower size particles can be taken up by Peyer's patches, and those lower than 1 µm can penetrate to lymph nodes and the liver, and reach the circulatory system.^{100,101} Liposomes, cochleates and microparticles can bind mucosal surfaces by hydrophobic interactions, but their entry to M cells is not efficient because they are rapidly trapped in mucosal gels and most of them are not able to reach the mucosa. Macromolecules or particles conjugated or covered by ligands such as cholera toxin b chain (CTB) are limited by their need to gain accessibility to specific receptors.¹⁰² The balance between hydrophobicity-hydrophilicity for the antigen delivery systems can be modified to obtain a modulation in the immune response.¹⁰⁰ The use of ligands linked to particles

can result in the specific adherence to M cells, but only in a size range restricted by the glycocalix. Particles of 1 µm or higher require targeting of ligands to M cells.¹⁰³

Living antigen mucosal delivery systems

Some pathogenic bacteria have the ability to overcome the difficulties of non-living systems in being uptaken easily by specific M cell receptors. One example is attenuated *Salmonella typhi* ty21a, which has a lectin-like interaction with polysaccharide receptors on M cells. Also *V. cholerae* and poliovirus strains can be used for oral immunization of heterologous antigens. Genetically modified strains of these microorganisms have been used as a carrier of heterologous antigens.¹⁰⁴ The biology of these living vectors introduces new challenges. *V. cholerae* vaccine strains without the genes coding for their toxins remain toxic.¹⁰⁴ Pre-existing immune responses in a high number of subjects previously immunized naturally or by vaccination constitutes a major drawback for this strategy.

Cytokines

High doses of IFN-α abrogate oral tolerance.¹⁰⁵ Similar results have been obtained with IL-12.¹⁰⁶ This suggests that orally administered cytokines may be able to be used as mucosal adjuvants to overcome systemic immune unresponsiveness, for example that seen in chronic Hepatitis B infection.

Adjuvants for DNA immunization

When naked DNA immunization commenced in the 1990s, it was supposed that this kind of immunogen would not need adjuvants. It is now clear that novel strategies are required to enhance the potency of DNA-based vaccine candidates. The strategy of co-inoculating plasmids coding for different cytokines or costimulatory factors to enhance the immune response generated by the vaccine plasmid has been used successfully.¹⁰⁷ Co-inoculation of the plasmid expressing B7-2 along with a DNA vaccine candidate from HIV-1, increased the cellular immune response specific for HIV-1. Also a plasmid expressing GM-CSF boosted the humoral immune response to protein G from rabies virus when two plasmids coding for each protein were co-inoculated.⁷³ IL-12 expressing plasmid co-inoculated along with another plasmid coding for an HIV-1 protein enhanced the cell mediated immunity specific for VIH-1.¹⁰⁸

DNA vaccines and particulate adjuvant systems

Polymers and particulate systems have been used in the field of DNA immunization. Polylactic microspheres, polycarbonates and polystyrene particles about 1 µm in size have been used mucosally and parenterally, resulting in better results compared to free DNA administration.¹⁰⁹ The use of mannans covering polymers of N t-butyl N' tetradecylamin-propionamidine (diC14 amidine), have been used as an immunoenhancing strategy for DNA vaccination. The main effects caused by the co-administration of these structures with DNA is the increase in DTH and CTL responses.

DNA vaccine immunomodulators of cancers have been used in DNA immunization. Ubenimex (UBX) increased humoral and cellular responses to DNA vaccination.¹¹⁰ The

immune response against DNA encoded antigens has been evaluated nasally and parenterally using immunomodulators already used with protein antigens like MPL and saponins.¹¹¹ The response found after intramuscular inoculation of DNA in PBS have been explained in part by the 'danger signal' offered by the DNA itself. Immunostimulatory sequences from procariotic DNA are able to induce several cytokines like IL-12, TNF and IL-6, and thereby have an adjuvant action.¹¹²

Cancer vaccine adjuvants

There is increasing excitement regarding the potential for anti-cancer vaccines to slow or even eradicate some tumours.^{113,114} These vaccines utilize either complete tumour cells, tumour antigens or tumour growth factor receptors combined with powerful adjuvants. These vaccines, being based on self molecules are generally of very low immunogenicity thereby leading to the requirement for potent adjuvants for effect. Approaches taken include the use of Montanide adjuvants, very small size proteoliposomes (VSSP) obtained from the external membrane of *Neisseria meningitidis*¹¹⁵ or the use of peptides adjuvated with GM-CSF.¹¹⁶

Conclusions

Despite an explosion of knowledge regarding immune function over recent decades, we remain almost totally reliant for human adjuvants on aluminium-based compounds whose activity was first discovered over 80 years ago. Recent advances in vaccine development and, in particular, the increasing use of recombinant subunit and synthetic vaccines makes the need for improved adjuvants all the more acute. Although there are glimmers of hope that new adjuvants may rectify some of the deficiencies of aluminium-based adjuvants, there remains a concern that many of these promising adjuvants will never be approved for human use for logistical or commercial rather than scientific reasons. Clearly there are some major barriers other than just a lack of scientific knowledge of adjuvants that are standing in the way of availability of new adjuvants. First and foremost, unacceptable side-effects and toxicity preclude the use of many candidate adjuvants and this is particularly true for prophylactic paediatric vaccines where safety issues are paramount. Second, the regulatory bar has been raised significantly since the days when alum was first introduced as a human adjuvant. Indeed, it is likely that if alum hadn't been in use all these years and was first put forward to regulatory bodies for approval today, it would be refused registration on the basis of safety concerns. Third, it is not possible for adjuvants to be approved as products in their own right as they can only be registered as part of a vaccine combination. It is possible that many good adjuvant candidates have failed to reach the registration phase, not because there were any problems with the adjuvants themselves, but because the vaccine combination was not effective or had toxicity. This could be seen as analogous to throwing out the baby with the bath water. Fourth, having invested considerable funds in the development of a new vaccine antigen, few companies are prepared to risk this investment by conducting clinical trial program of candidate antigens with a new and unproven adjuvant as this

could bring the whole development program unstuck if there turned out to be problems with the adjuvant. Fifth, most vaccine companies choose to keep their proprietary adjuvant data secret and therefore until such time as they themselves wish to register a vaccine product based on their adjuvants, then they will not share their knowledge of these adjuvants with others. Finally, the cost of developing a new product such as an adjuvant is now prohibitive. Whilst it might be possible to justify an investment of several hundred million dollars on a new vaccine given the prospect of recovering this investment from vaccine sales, the same does not hold true for adjuvant development costs, for which there is no easy source of cost recovery. For all the above logistical and commercial reasons there is a continuing major unmet need for safe and non-toxic adjuvants, particularly for adjuvants capable of strongly boosting cellular immune responses which are not associated with undue toxicity. Despite many advances of immunology, this key objective remains the 'holy grail' of vaccinology. Hence, the importance of major public institutions such as the NIH and WHO and charities with interests in vaccine development such as the Gates Foundation to fund adjuvant research and development programs as part of their general support for vaccine development.

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Safety and Immunogenicity of Increasing Doses of a *Clostridium difficile* Toxoid Vaccine Administered to Healthy Adults

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Clostridium difficile is a major cause of nosocomial diarrhea in industrialized countries. Although most illnesses respond to available therapy, infection can increase morbidity, prolong hospitalization, and produce life-threatening colitis. Vaccines are being explored as an alternative means for protecting high-risk individuals. We assessed the safety, immunogenicity, and dose response of a parenteral vaccine containing *C. difficile* toxoids A and B. Thirty healthy adults were assigned to receive four spaced inoculations on days 1, 8, 30, and 60 with one of three doses of vaccine (6.25, 25, or 100 µg). At each dose level, subjects were randomized, in a double-blind fashion, to receive either the soluble toxoids ($n = 5$) or toxoids adsorbed to alum ($n = 5$). Subjects were monitored for clinical and immunologic responses to vaccination. Vaccination was generally well tolerated, with occasional, usually mild, systemic reactions (abdominal pain, arthralgia, and diarrhea). The most common local reaction, mild arm pain, was reported by all recipients of the toxoid-alum formulation. Nearly all subjects ($\geq 90\%$) developed vigorous serum antibody responses to both toxins, as measured by immunoglobulin G (IgG) enzyme-linked immunosorbent assay and neutralization of cytotoxicity, whereas fecal IgA increases occurred in approximately 50%. Statistically significant effects of dose and formulation on immunogenicity were not seen, although antibody levels tended to be higher with the alum-adjuvanted formulations and with increasing doses of soluble toxoid. Serum antibody responses among the toxoid-alum group appeared to plateau at 25 µg. We concluded that the *C. difficile* toxoid vaccine is safe and immunogenic in healthy volunteers. Further development as a prophylactic vaccine or for producing *C. difficile* hyperimmune globulin is justified.

Clostridium difficile is the major infectious cause of nosocomial diarrhea in industrialized countries (1, 24) and a well-recognized source of recalcitrant diarrheal outbreaks, particularly affecting units for the elderly (26) and immunocompromised (4). In a recent study, it was found that 17% of patients who are hospitalized for 2 or more days and receive antibiotics will develop *C. difficile*-associated diarrhea (CDAD) (19). Reports from several countries suggest that the incidence of CDAD has been rising in recent years (10, 38). Although most illnesses resolve with discontinuation of antibiotic therapy and provision of specific antibiotic treatment, these infections may prolong hospitalization (14), inflate medical costs (28, 31), increase morbidity (21), and produce severe, life-threatening colitis (11). Furthermore, there are drawbacks to antimicrobial treatment for CDAD, including secondary infection with multiresistant organisms (29) and clinical relapse, which occurs in 5 to 30% of patients after antibiotic therapy is discontinued (9, 25). Innovative approaches for prevention and treatment are thus needed, particularly those that reduce the need for antibiotic therapy (9).

The pathogenesis of CDAD is mediated by the actions of

two protein exotoxins, toxin A and toxin B, which induce mucosal injury and inflammation of the colon (17, 27). Strategies that provide antibody-mediated antitoxic immunity are being explored. An effective vaccine could be used to elicit protective immunity in individuals at high risk for CDAD or to produce hyperimmune globulin preparations as a means for passive immunoprophylaxis or treatment. Herein we report the results of a phase 1 study to assess the safety, immunogenicity, and dose response, in healthy adults, of a parenteral vaccine containing *C. difficile* toxoids A and B with and without alum adjuvant.

MATERIALS AND METHODS

Vaccine manufacture and administration. The formalin-inactivated toxoid A and B vaccine was produced using good manufacturing practices at the Centre for Applied Microbiological Research, Porton Down, United Kingdom. Anaerobic cultures of *C. difficile* (strain ATCC 43255) were grown in dialysis sacs suspended in growth medium. Viable *C. difficile* and spores were removed from pooled sacs by centrifugation followed by submicron filtration, the filtrate was concentrated and diafiltered, and then the toxin was precipitated at 4°C with 60% ammonium sulfate and the pellets were frozen. At Forest Glen (building 501), Walter Reed Army Institute of Research, the pellets were dissolved in phosphate buffer and applied to an S300 Sephacryl size exclusion column. The peak containing both toxin A and toxin B was collected, concentrated, and then inactivated for 18 days with formaldehyde at 4.25 mg/ml at 4 to 6°C in a solution containing lysine at 4.25 mg/ml. The inactivation period exceeds three times the interval needed for complete elimination of in vivo biological activity (lethality in mice). After formalin treatment, activity in the IMR90 cytopathology cell culture assay was at the threshold of detectability (cytotoxicity reduced by 8 to 9 log₁₀). The formaldehyde concentration was then reduced by diafiltration to 0.016%, thus retaining a low concentration to prevent toxoid reversion.

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TABLE 1. Study design^a

Cohort no.	Dose of toxoid (μg)	Randomized assignment	No. of subjects
1	6.25	Toxoid	5
		Toxoid-alum formulation ^b	5
2	25	Toxoid	5
		Toxoid-alum formulation ^b	5
3	100	Toxoid	5
		Toxoid-alum formulation ^b	5

^a Vaccine was administered as an i.m. injection into the deltoid muscles of alternating arms on days 1, 8, 30, and 60.

^b 723 to 757 μg of alum.

The final product (lot no. 98F03) was used to fill glass vials at a volume of 0.6 ml and stored at 2 to 8°C. The total protein concentration was 0.52 mg/ml, of which toxins A and B comprised about 44% (by gel densitometry) at a 1.5:1 toxin A to toxin B ratio (as measured by capture enzyme-linked immunosorbent assay [ELISA]). The final product passed safety tests developed at OraVax (Cambridge, Mass.) for mouse toxicity (0.5-mg intraperitoneal dose) and cytotoxicity (up to 0.25 mg/ml) in IMR90 cells. In addition, no toxicity occurred when hamsters were injected with four intramuscular (i.m.) doses of 500 μg/kg (with and without alum) or when cynomolgus monkeys were given either a single i.m. dose of 20 μg/kg (with and without alum) or a single i.m. dose of 2,700 μg/kg without alum. The potency of the final product was tested by inoculating, via intraperitoneal injection on days 1 and 7, groups of eight 10- to 12-week-old mice, using graded doses of toxoid adsorbed to alum. Mice were bled on day 14 to determine the 50% effective dose, which was defined as the toxoid dose that elicited a toxin A and B neutralizing antibody titer of ≥1:25 (a titer that protects mice against intraperitoneal challenge with 10 50% lethal doses of toxins A and B). The vaccine lot had a 50% effective dose comparable to that of a reference control (a preclinical lot of vaccine manufactured by the same process as the clinical lot). Using this reference control lot, the stability of the toxoid state at 37°C was verified by the IMR90 cytotoxicity assay and mouse toxicity for 3 months and, in a second series of studies, by the IMR90 cytotoxicity assay for 300 days at 4, 28, and 37°C. The clinical protocol was conducted under an approved investigational new drug application (BB-IND 7932) filed with the U.S. Food and Drug Administration.

On the morning of vaccination, the investigator performed serial dilutions using phosphate-buffered saline (PBS) with 0.016% formaldehyde (pH 7.4) to achieve the desired dose in 0.5 ml. The final dose (6.25, 25, or 100 μg) represented the total protein content, of which toxoids A and B represented 44%, at a ratio of 1.5 to 1. Rehydralg aluminum hydroxide adjuvant (Reheis; 723 to 757 μg) was added to half of the vaccine doses as specified by the randomization scheme described below. Vaccine was administered by an unblinded nurse who was not involved in subsequent clinical assessment of volunteers.

Recruitment, study design, and clinical evaluation. (i) Recruitment and medical screening. Healthy adults 18 to 50 years of age were recruited from the Baltimore-Washington community and the University of Maryland College Park campus. After medical history determination, physical examination, and a battery of laboratory tests, individuals meeting the following criteria were excluded: significant medical or psychiatric abnormalities, a history of gastrointestinal disease including previous antibiotic-associated diarrhea, a positive fecal *C. difficile* stool cytotoxin assay, antibiotic therapy during the previous month, and failure to achieve a passing grade (70%) on a written examination which tested the volunteer's comprehension of the trial. Women were required to have a negative pregnancy test within 7 days of each vaccination. Volunteers who satisfied all eligibility requirements and provided informed, written consent in accordance with the guidelines of the Institutional Review Boards at the University of Maryland in Baltimore and in College Park were enrolled in the study.

(ii) Allocation and vaccination procedures. Volunteers were sequentially assigned, in groups of 10, to receive one of three increasing doses of vaccine (6.25, 25, or 100 μg) as shown in Table 1. Five subjects at each dose level were randomized, in a double-blind fashion, to receive soluble toxoid vaccine, and five subjects were randomized to receive an equivalent dose of toxoid adsorbed to alum. The regimen consisted of four inoculations administered by i.m. injection into the deltoid muscle (alternating arms) on study days 1, 8, 30, and 60.

To increase the margin of safety, initial exposure to each dose of vaccine was limited by vaccinating two subjects in each cohort 1 week before the remaining

eight subjects. While maintaining the double-blind condition; one subject received the soluble toxoid and the other received toxoid with alum. These two subjects were observed for 1 week for clinical tolerance before the remaining eight subjects were inoculated at that dose level.

(iii) Clinical monitoring. Subjects were monitored at the study site for 30 min after receiving vaccine to detect immediate reactions. For 7 days after each inoculation, they kept a diary in which they recorded their evening oral temperature; the time, date, and consistency (loose or formed) of all stools passed; and the following signs and symptoms: anorexia, malaise, joint swelling, numbness or tingling in fingers or toes, rash, abdominal pain, joint pain, injection site pain, and other, unsolicited complaints. Subjects graded their pain as mild (aware of symptom but symptom easily tolerated), moderate (symptom interferes with usual activity), or severe (symptom prevents work or ability to perform usual activity). In addition, a medical history was obtained at each visit to the study site and by telephone interview 3 days after each inoculation. A panel of clinical laboratory tests (complete blood count with differential and platelet counts, creatinine, alanine aminotransferase, and urinalysis) was performed 1 week after each inoculation.

Study personnel inspected the injection site 1 and 7 days after each inoculation for the presence of local reactions (swelling, erythema, or heat). Swelling and erythema were graded (maximum diameter) as mild (≤4.0 cm), moderate (5.0 to 6.0 cm), or severe (≥7.0 cm).

Laboratory methods. (i) ASC responses. Peripheral blood mononuclear cells (PBMC) were collected before and 7 days after administration of each dose of vaccine to measure circulating immunoglobulin A (IgA) and IgG antibody-secreting cells (ASC) recognizing toxin A and toxin B, by adapting a previously described ELISPOT assay (33). Briefly, microdilution plates were coated with either toxin A (5 μg/ml) or B (1 μg/ml) antigen (lots VC011199 and 06986006, respectively, obtained from culture filtrates, highly purified by anion-exchange chromatography, and supplied by OraVax). Samples from each subject at each time point were run at initial concentrations of 2.5×10^5 PBMC/well and at dilutions of 1:10 and 1:100 for each immunoglobulin isotype and antigen, in quadruplicate. Negative controls (uncoated plates) were run for each subject and assay, and the values were subtracted from the observed response. Positive controls (antitoxin A IgG2a-producing PCG-4 mouse hybridoma, lot 8/26/98, obtained from OraVax) were run on each day of testing. A positive ELISPOT response was defined as a geometric mean postvaccination count per 10^6 PBMC ≥3 standard deviations above the mean prevaccination count, in the log metric. Actual cutoff values designating a positive response for antitoxin A were 3.7 (IgA) and 1.2 (IgG) ASC per 10^6 PBMC, and for antitoxin B they were 0.9 (IgA) and 0 (IgG) ASC per 10^6 PBMC.

(ii) Serum antibody responses. Sera were collected prevaccination and 8, 15, 30, 37, 60, 67, and 90 days after immunization and tested for antibodies to toxins A and B by ELISA and by neutralization assay. To detect neutralizing antibody, IMR90 cells were seeded 1 day prior to use into microtiter plates at 1.8×10^4 cells/well in Eagle minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum and 1% L-glutamine (EMEM-10). The toxins were diluted to eight times their 50% cytotoxicity titer for use in the assay. An equal volume of serum, diluted 1:5 in triplicate using EMEM-10 and titrated by serial twofold dilutions, was added to the diluted toxin and placed at 37°C for 1 h. Medium was then removed from plates and replaced with 100 ml of the serum-toxin mixture. The cell plates containing the serum-toxin mixtures, along with positive and negative controls, were incubated for 16 to 20 h in a 37°C, 5% CO₂ incubator. The percentage of cells rounded was scored as 0 to 4 (0 = 0 to 10% of cells rounded, 1 = 11 to 39% rounded, 2 = 40 to 60% rounded, 3 = 61 to 89% rounded, and 4 = 90 to 100% rounded). The final data were reported as the 50% neutralizing titer (highest serum dilution at which the rounding score was ≥2). Seroconversion was defined as a fourfold or greater rise in titer following vaccination.

To detect specific IgA and IgG by ELISA, plates were coated with toxin antigens as described above for the ELISPOT assay, stored overnight at 4°C, washed with PBS, and then blocked using PBS with 5% fetal bovine serum (FBS). Eight serial twofold dilutions of the following were run, in duplicate, in PBS-Tween with 1% FBS: each serum specimen starting at 1:100, control wells with no antigen, and the IgG and IgA reference standards starting at 1:200 for the IgG reference (IVIG [Baxter, Deerfield, Ill.], containing anti-toxin A and B antibody at 50,000 ELISA units [EU]/ml) and the IgA reference (IgAbulin [OraVax], containing anti-toxin A and B antibody at 100,000 EU/ml). After incubation and washing, alkaline phosphatase-labeled goat anti-human IgG or IgA antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted in PBS with 1% FBS was added. The plates were incubated and washed, substrate was added, and the standard optical density at 500 EU/ml was monitored. The reaction was stopped when the IgG or IgA reference reached maximum antibody

TABLE 2. Systemic complaints reported in a standardized diary within 7 days following each inoculation with *C. difficile* toxoid vaccine, by dose and formulation

Complaint	No. of subjects ^a who received:					
	6.25 µg of toxoid		25 µg of toxoid		100 µg of toxoid	
	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum
Anorexia	1	0	0	0	1	1
Malaise	2	0	0	2	1	0
Joint swelling	0	0	0	0	0	1
Numbness	1	0	0	0	1	1
Rash	1	2	0	2	2 ^b	1 ^b
Abdominal pain	1 ^c	1	1	0	2	1
Joint pain	0	1	0	1	0	0
Fever	0	0	0	0	0	0
Diarrhea	1 ^c	0	0	0	1 ^b	0
Any of the above	3	2	1	3	2	2

^a There were five subjects in each treatment group.

^b It should be noted that one subject in each group experienced two episodes of the specified complaint (after different inoculations).

^c This subject experienced three episodes of the specified complaint (after different inoculations).

binding as indicated by predetermined maximal absorbance values of the 1:100 and 1:200 dilutions of the IgG and IgA standards. Serum samples were read at a dilution corresponding to the linear portion of the run's reference curve after it was ascertained that the run's reference curve and the standard reference curve absorbance values had a correlation, *r*, of ≥ 0.9 . Limits of detection of anti-toxin A and anti-toxin B IgG were 31.25 and 62.5 EU/ml, respectively; limits of detection of anti-toxin A and anti-toxin B IgA were 7.81 and 15.62 EU/ml. Seroconversion was defined as a fourfold or greater rise in ELISA units following vaccination.

(iii) **Fecal antibody responses.** Specific fecal IgA antibody responses were measured before and 7 days after each dose of vaccine by ELISA using previously described assays adapted for toxin A and B antigens (22). Briefly, volunteers submitted a fresh (<24 h old) stool sample, which they maintained in an insulated bag containing a cold pack. A 10% stool supernatant containing protease inhibitors was assayed for total IgA using a reference standard of purified IgA from human colostrum and then standardized to 2 mg% total IgA to ensure that specific antibody would be detected at levels of $\geq 0.25\%$ of the total IgA. The anti-toxin A- and B-specific IgA ELISAs were performed as described above for serum IgA, except that the reference standard used was the colostrum IgA reference curve and results were reported as nanograms of specific antibody per milliliter. This method of quantifying antibodies is based on the equivalence of absorbances between a reference ELISA and anti-toxin ELISA performed in parallel under identical laboratory conditions using the same buffers, temperatures, incubations, enzyme conjugates, and reagent dilutions (39). A response was defined as a fourfold increase in titer postvaccination.

Statistical methods. One-tailed Fisher's exact tests were performed to determine whether alum increased the proportion of subjects (in each randomized group of five) that experienced systemic or local reactions; linear regression analyses were used to assess whether alum amplified the severity of these reactions. Analyses were performed independently for each formulation to evaluate whether reactions were more frequent (Cochran-Armitage tests for trend) or more severe (regression analyses) at higher doses. To assess whether either alum or dose level enhanced the immune response, separate logistic regression analyses were performed for each immunologic assay, with response as the dichotomous dependent variable; analysis of variance (ANOVA) was used to determine whether alum or a dose increase increased the magnitude of these responses. For both logistic and ANOVA models, the independent variables also included the interaction between alum and dose level. Relationships among preimmunization and peak postimmunization titers and among peak postvaccination titers for the various immunologic assays were examined using Spearman's correlations. Statistical significance was assessed at the 5% level.

RESULTS

Description of study subjects. The median age of the 30 subjects was 23 years; 23 (77%) were men and seven (23%)

were women. The distribution by race and ethnicity was as follows: Caucasian, 47%; African American, 33%; Hispanic, 3%; and Asian or Pacific Islander, 17%. All 30 subjects enrolled received four doses of vaccine and were included in the analysis.

Clinical tolerance. (i) Systemic reactions. There were no serious adverse events during the study, and the vaccine was generally well tolerated (Table 2). No subject experienced fever. The most common complaint was rash (eight subjects), which uniformly was localized, did not involve urticaria or petechiae, and usually could be attributed to a cause other than vaccination, such as contact dermatitis. Six subjects reported eight episodes of abdominal pain; most episodes were rated mild ($n = 5$) or moderate ($n = 2$), lasting 3 or fewer days, although one subject experienced fleeting (5-min duration) abdominal pain on 2 nonconsecutive days that he rated severe. Two subjects noted arthralgia for 1 to 3 days; one attributed the pain to muscle soreness after vigorous exercise, and the other attributed it (elbow pain) to the contiguous tender injection site. Two subjects experienced five episodes of non-bloody diarrhea (defined as three or more loose stools within 24 h), with passage of three to eight loose stools. There were no statistically significant differences in the frequency, duration, or severity of systemic reactions between dosage groups or between formulations (alum versus soluble toxoid). Clinically significant laboratory abnormalities that appeared to be vaccine related were not seen.

(ii) **Local reactions.** Pain at the site of injection was the most frequent local reaction, occurring in 100% of subjects who received toxoid adsorbed to alum, compared with zero, two, and one of the five subjects who received soluble toxoid, at each increasing dose level (Table 3). Most episodes of pain (55%) lasted for a single day, and all resolved within 4 days. Whereas no subject experienced local heat, self-limited swelling or erythema was observed at the two higher doses (Table 3). All subjects graded their local reactions as mild, except for one recipient of 25 µg of toxoid with alum who experienced mild-to-moderate arm pain after the third vaccination and moderate-to-severe pain after the fourth dose. Recipients of alum were more likely to experience arm pain ($P = 0.004$, 0.08, and 0.02 for the 6.25-, 25-, and 100-µg dose groups, respectively). The frequency of local swelling ($P < 0.001$ in the alum

TABLE 3. Local reactions occurring within 7 days following administration of each dose of *C. difficile* toxoid vaccine, by dose and formulation^a

Reaction	No. of subjects ^b who received:					
	6.25 µg of toxoid		25 µg of toxoid		100 µg of toxoid	
	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum
Pain	0	5	2	5	1	5
Swelling	0	0	0	1	1	4
Erythema	0	0	0	1	3	3
Heat	0	0	0	0	0	0
Any local reaction	0	5	2	5	3	5

^a The reactions were solicited in a systematic fashion. In addition, six of the subjects spontaneously reported pruritus at the injection site after receiving either 6.25 µg of toxoid (two subjects), 25 µg of toxoid-alum (two subjects), 100 µg of toxoid (one subject), or 100 µg of toxoid-alum (one subject).

^b There were five subjects per treatment group.

TABLE 4. Immune response to *C. difficile* toxoid vaccine

Assay	No. of subjects with response/total (geometric mean peak postvaccination assay result)					
	6.25 µg of toxoid		25 µg of toxoid		100 µg of toxoid	
	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum	Toxoid	Toxoid-alum
Neutralizing antibody						
Anti-toxin A	4/5 (80) ^a	5/5 (269)	5/5 (134)	5/5 (604)	5/5 (339)	5/5 (243)
Anti-toxin B	4/5 (368)	4/5 (80)	5/5 (368)	5/5 (1,513)	5/5 (485)	5/5 (2,560)
Serum antibody ELISA						
Anti-toxin A						
IgA	4/5 (238) ^b	5/5 (785)	5/5 (1,622)	5/5 (5,272)	5/5 (6,369)	5/5 (1,548)
IgG	5/5 (275)	5/5 (1,217)	5/5 (422)	5/5 (2,345)	5/5 (1,060)	5/5 (1,260)
Anti-toxin B						
IgA	4/5 (13)	3/5 (4)	3/5 (13)	5/5 (39)	5/5 (29)	5/5 (23)
IgG	4/5 (56)	5/5 (64)	3/5 (161)	5/5 (488)	5/5 (183)	5/5 (342)
ASC ELISPOT						
Anti-toxin A						
IgA	4/5 (12) ^c	3/5 (6.7)	5/5 (23)	5/5 (84)	5/5 (179)	5/5 (89)
IgG	4/5 (5.2)	2/5 (2.8)	5/5 (30)	4/5 (79)	4/5 (38)	5/5 (63)
Anti-toxin B						
IgA	3/5 (6.6)	5/5 (5.7)	5/5 (60)	5/5 (86)	5/5 (134)	5/5 (115)
IgG	5/5 (5.4)	5/5 (1.8)	5/5 (54)	5/5 (170)	5/5 (42)	5/5 (123)
Fecal antibody ELISA						
Anti-toxin A IgA	5/5 (2,219) ^d	4/5 (5,275)	1/4 (907)	0/5 (779)	2/4 (329)	2/3 (210)
Anti-toxin B IgA	2/5 (43)	3/5 (60)	2/4 (170)	2/5 (127)	3/4 (157)	3/3 (95)

^a Reciprocal titer.^b 10³ EU/ml.^c Number of ASC per 10⁶ PBMC.^d Nanograms per milliliter.

formulation) and erythema ($P = 0.04$ and 0.02 in the formulations with and without alum, respectively) increased with increasing doses of vaccine.

It is notable that six subjects reported pruritus (without urticaria) at the site of injection, even though this symptom was not specifically solicited. These responses were seen with equal frequency at all dose levels (2 of 10 subjects per dose group) and among subjects who received the alum-adjuvanted formulation and those who did not (3 of 15 subjects per formulation). The onset was 1 to 6 days after injection in five subjects and 30 min after inoculation in the sixth, with a duration of 1 to 4 days. Pruritus was experienced by three subjects after the second injection only, by two after the third injection only, and by one after the second and third injections.

Immune responses. (i) Serum neutralizing antibody responses. The vaccine elicited fourfold rises in neutralizing antibody to toxins A and B in all but two subjects in the 6.25-µg group (one received alum, and one did not). For toxin A, the highest geometric mean titers followed the 25-µg dose of toxoid-alum formulation and the 100-µg dose of toxoid alone; for toxin B, the titers consistently increased with increasing doses (Table 4 and Fig. 1). In most instances, the responses following toxoid-alum administration exceeded those following soluble-toxoid administration. Nonetheless, statistically significant effects of dose and formulation on the magnitude of the responses were not seen.

(ii) Serum antibody responses determined by ELISA. Serum anti-toxin A IgA and IgG antibody responses were seen in all

but one subject (who received 6.25 µg of soluble toxoid; Table 4). The magnitude of the anti-toxin A responses was high (14 of 30 or 47% of IgA responses and 17 of 30 or 57% of IgG responses were at least 10⁶ EU/ml). Among recipients of soluble toxoid, the antitoxin A and B responses increased with the dose (although not significantly); however, responses among the toxoid-alum group appeared to plateau after 25 µg (Table 4 and Fig. 2). Accordingly, the serum antibody responses following toxoid-alum administration generally exceeded those following soluble-toxoid administration for the 6.25- and 25-µg dose groups but not for the 100-µg group, although no significant effect of formulation on the magnitude of the response was revealed by multivariate ANOVA.

(iii) Kinetics of serum antibody responses. Although a rise in antibody titer generally followed each injection of vaccine, the fourth dose did not substantially enhance the overall anti-toxin A antibody levels in the two higher-dose groups (Fig. 1 and 2). For anti-toxin B, the fourth dose elicited a booster effect on IgG antibody in a neutralizing assay (Fig. 1) but not in an ELISA (Fig. 2). There was a strong correlation between levels of serum neutralization, which is the assay likely to predict levels of physiologic antibody, and serum anti-toxin A IgG, which has been shown to be a correlate of protective immunity ($r = 0.83$, $P < 0.001$) (19).

To explore the feasibility of preventing primary or relapsing CDAD by administering this vaccine to patients upon admission to the hospital or after an episode of CDAD, we determined the proportion of subjects whose anti-toxin A IgG an-

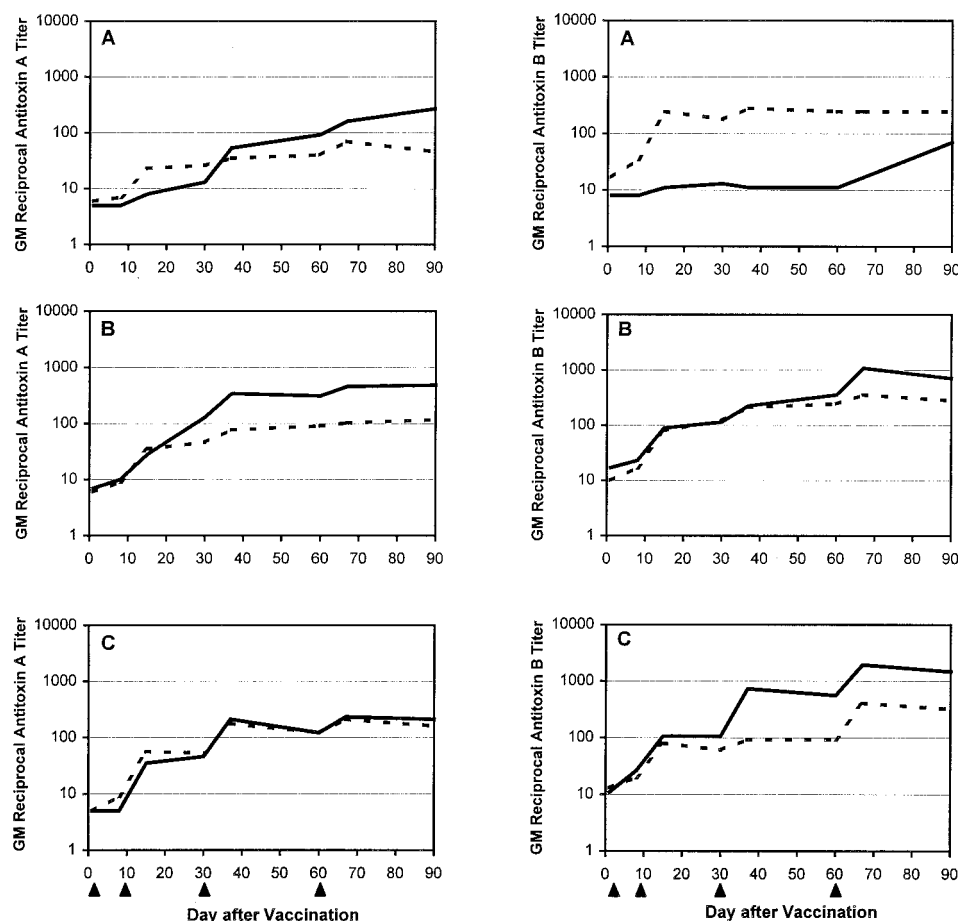


FIG. 1. Geometric mean (GM) serum anti-toxin A and anti-toxin B neutralizing antibody titers among volunteers who were inoculated with *C. difficile* toxoid vaccine at a dose of either 6.25 (A), 25 (B), or 100 (C) μ g. At each dose, five subjects received soluble toxoid vaccine (broken line) and five received alum-adsorbed toxoid (solid line). Each subject received a dose of vaccine on days 1, 8, 30, and 60 (arrowheads).

titers rose at least fourfold by day 8 (1 week after administration of the first dose) and day 15 (1 week after administration of two doses). Only two subjects (both received 100 μ g of toxoid-alum) mounted a fourfold rise in antibody by day 8. Whereas all five subjects who received 100 μ g of toxoid-alum seroconverted by day 15, with the fold rise ranging from 13 to 403, a 4-fold response occurred in only two or three subjects in each of the other dose groups. Seroconversion to toxin B was also infrequent by day 8 (three subjects) but common by day 15, occurring in all of the subjects who received 100 μ g of toxoid (10- to 196-fold rises), in four of five subjects who received 100 μ g of toxoid-alum formulation (30- to 107-fold rises), and in two to four subjects in each of the other dose groups (4- to 42-fold rises). Early neutralizing antibody seroconversions were less common (data not shown).

(iv) ASC responses. Vaccination elicited an IgA or IgG ASC response to toxin A in 27 (90%) and 24 (80%) subjects and to toxin B in 28 (93%) and 30 (100%) subjects, respectively (Table 4). All but one subject in the two highest-dose groups responded in all four assays. There was a trend toward increasing magnitude of response with increasing doses in each of the assays, which achieved statistical significance only for anti-toxin A IgA ($P = 0.02$) and anti-toxin B IgG ($P < 0.01$). The

height of the response was not significantly different between the two formulations ($P > 0.47$).

(v) Fecal antibody responses determined by ELISA. Fecal IgA responses occurred less frequently than did the other immune responses measured (Table 4). There were no significant differences between the groups or the two formulations.

DISCUSSION

We found that a *C. difficile* vaccine containing formalin-inactivated toxoids A and B was safe, without serious adverse reactions, and highly immunogenic in healthy volunteers, even at doses as low as 6.25 μ g of total protein (containing 1.5 μ g of toxoid A and 1.1 μ g of toxoid B). Vaccination elicited systemic antibody responses in nearly all subjects, in both binding (ELISA) and physiologic (neutralization) assays. The dose-response studies indicated that the maximum response occurred after administration of 25 μ g of the toxoid-alum formulation and after administration of 100 μ g of toxoid alone. Three doses of vaccine (days 1, 8, and 30) seem to be sufficient, since the fourth dose did not appear to substantially boost serum IgG or neutralizing antibody.

Considerable evidence supports the role of antibody-mediated

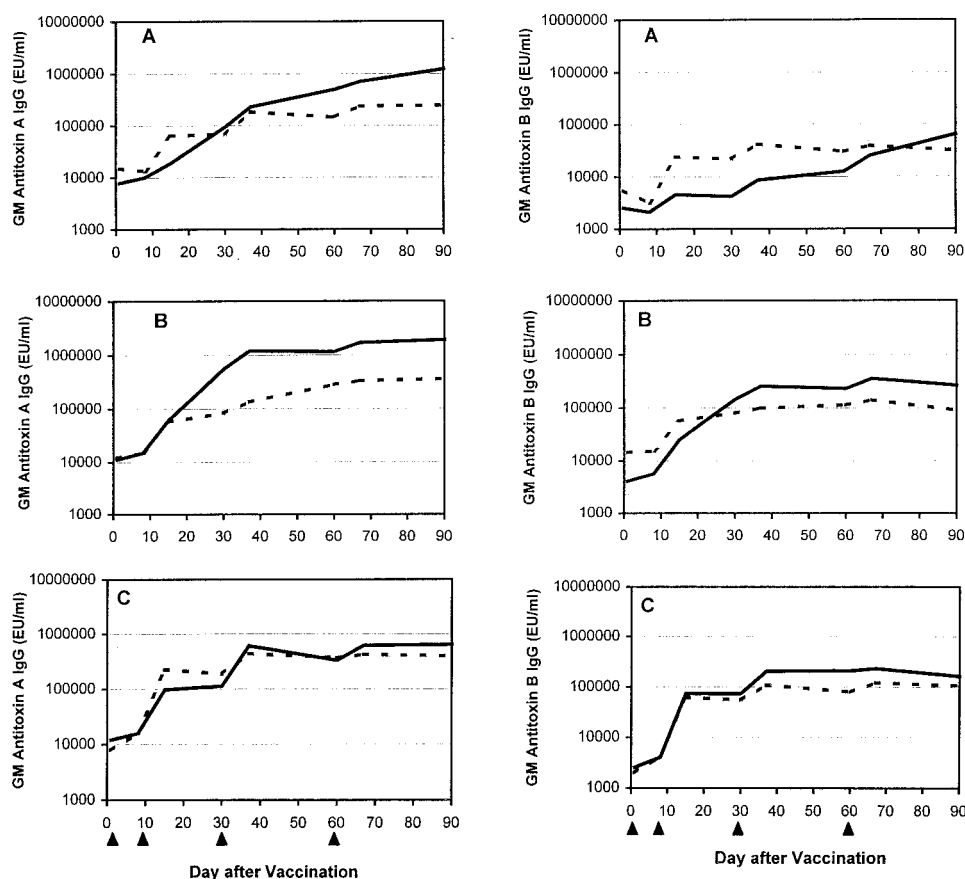


FIG. 2. Geometric mean (GM) serum anti-toxin A and anti-toxin B IgG responses, as measured by ELISA, among volunteers who were inoculated with *C. difficile* toxoid vaccine at a dose of either 6.25 (A), 25 (B), or 100 (C) µg. At each dose, five subjects received soluble toxoid vaccine (broken line) and five received alum-adsjuvanted toxoid (solid line). Each subject received a dose of vaccine on days 1, 8, 30, and 60 (arrowheads).

ated anti-toxic immunity in protection and recovery from *C. difficile*-associated disease. In animal models of *C. difficile* infection, parenteral and mucosal administration of specific toxin-neutralizing monoclonal and polyclonal antibodies prevents experimentally induced *C. difficile*-associated enterotoxicity (3, 6, 16, 23). Furthermore, treatment with toxin-neutralizing oral IgY preparations (derived from egg yolk harvested from immunized hens) effectively averts CDAD (16). In addition, active immunization with toxoid preparations, delivered either parenterally or by a combination of the parenteral and mucosal routes, protects animals against diarrhea and death following a challenge with *C. difficile* (5, 6, 32).

Data from humans also support a role for anti-toxic immunity in protection against CDAD. In a prospective study of hospitalized patients who were receiving antibiotics, Kyne et al. showed that patients who developed diarrhea after becoming colonized with *C. difficile* had significantly lower levels of serum anti-toxin A IgG at the time of colonization compared with patients who remained asymptomatic (19). In another study, patients with low anti-toxin A titers in serum (IgG) and feces (IgA) were more likely to experience prolonged and relapsing CDAD (37). Several case reports have described patients with severe, persistent, or relapsing CDAD who were effectively treated with preparations of intravenous gamma globulin containing *C. difficile* antitoxin (20, 30, 36).

In light of these observations that serum anti-toxin A IgG antibody levels correlate with protection against CDAD (16, 19), it has been proposed that this measure be used as a surrogate marker of vaccine efficacy (19). Thus, it is important to note that in our trial, vaccination elicited a serum IgG antibody response to toxin A in all of the subjects and that the functional activity of these antibodies could be demonstrated by neutralization of toxin-induced cytotoxicity in all but one subject (who belonged to the lowest-dose group). Furthermore, the responses were strong, with the median fold increases in anti-toxin A antibody levels 90 days after administration of the first dose of vaccine ranging from 32 to 43 for neutralization and from 42 to 92 for IgG. It is likely that many of these responses were anamnestic, since most of the subjects had measurable baseline serum IgG antibody levels. Other studies have detected serum antibodies to *C. difficile* toxins in approximately 60% of the children and adults tested in the United States (34), presumably as a result of asymptomatic colonization during infancy and early childhood (18, 35).

Whereas serum anti-toxin A IgG in the ELISA has been correlated with immunity, the protective function of other immune responses is less clear. In the hamster model, protection against death following a challenge with *C. difficile* correlates more closely with *in vitro* neutralization of toxins A and B in a cell-rounding (cytotoxicity) assay than with anti-toxin antibody

levels measured by ELISA (6). The relative importance of anti-toxin A and B antibody in preventing intestinal damage is also not known. Immunological data which show an inverse correlation between anti-toxin A antibodies and disease expression (19) suggest that toxin A is the principal culprit, while studies of toxin binding to human colonic epithelium indicate that toxin B mediates disease and must be targeted for immunoprotection (27). Stimulation or transfer of antibodies to both toxins should therefore be a goal for vaccination and passive immunotherapy.

Mucosal antibody responses, although less common than systemic antibody responses, were also present in about half of the vaccinees. A possible role for mucosal immunity in protection from and treatment of CDAD has been postulated. The presence of secretory IgA antibodies in colonic aspirates from patients which inhibit binding of toxin A to its glycoprotein receptor in rabbit ileal intestinal brush border membranes suggests a potential mechanism of action (12). Further support for a role of secretory IgA in immunity to *C. difficile* comes from observations of protective efficacy conferred by orally administered antitoxic immunoglobulin preparations in animals (15, 16), the ability of bovine colostrum concentrate containing antibody to toxins A and B to neutralize the biologic effects of *C. difficile* toxins (cytotoxicity in cultured human fibroblasts, binding of toxin A to its enterocyte receptor, increased fluid secretion and mannitol permeability in rat ileal loops) (13), and the clinically milder illness seen in patients who have high fecal anti-toxin A IgA titers (37). The enterotoxicity of *C. difficile* toxins has been attributed to their ability to disrupt the actin cytoskeleton and enhance tight-junction permeability in human intestinal epithelial cell monolayers (7), since in vivo, these effects could result in transudation of fluid into the intestinal lumen and watery diarrhea. This pathogenesis may explain the protective activity of parenterally administered vaccines and antibodies, since access of serum antibodies to the intestinal lumen would be an early event in infection.

Several potential uses can be envisioned for a *C. difficile* vaccine. Plasma donors could be immunized to generate hyperimmune globulin for passive immunoprophylaxis of selected high-risk subjects, such as hospitalized patients; those with a high (circa 17%) risk of CDAD can be identified, based on factors such as age, anticipated hospital stay, antibiotic treatment, admission to a ward where *C. difficile* is endemic, and severity of underlying illness (19). Passive immunoprophylaxis might also benefit patients with multiple relapses of CDAD, particularly since data suggest that they have an underlying inability to mount an effective humoral immune response to infection (37). Hyperimmune globulin as a potential treatment of patients with established CDAD is another strategy which warrants further investigation. Compared with the large doses of IVIG that have been used to treat chronic relapsing CDAD (20, 30, 36), hyperimmune globulin would allow optimization of the antibody dose using a lower volume of product.

If the immune response to vaccination were rapid, then active vaccination of high-risk subjects upon admission to the hospital might be feasible. The decision to administer the first two doses of vaccine within a 1-week interval was made with this approach in mind. Unfortunately, few subjects mounted responses within 1 week, although all recipients of 100 µg of

the toxoid-alum formulation mounted at least a 13-fold increase in serum IgG anti-toxin A antibody within 14 days of receiving the first vaccination.

Primary prevention of CDAD by vaccination of groups with a high incidence of disease, such as adults 65 years of age or older, is another potential application. In Sweden, for example, the incidence of CDAD in adults between 60 and 99 years of age is 121 to 526 per 100,000 population (10). Interestingly this compares with 11 to 16 per 100,000 for invasive pneumococcal disease among the elderly in Sweden (2). With this approach, vaccine would be administered when individuals are in a relatively healthy state and more likely to respond immunologically. Furthermore, prevention of community-acquired illness would be possible. Population-based analyses indicate that a substantial proportion of CDAD occurs in the community. In a Swedish study, 28% of CDAD cases were community acquired, yielding an annual incidence of 20 per 100,000 population (with 56% of patients requiring hospitalization) (10), whereas in a Boston health maintenance organization, the incidence was 7.7 per 100,000 participants (with 18% of subjects hospitalized) (8).

For any of these clinical applications, the vaccine must be well tolerated, as well as immunogenic. In our study, occasional systemic reactions (abdominal pain, arthralgia, and diarrhea) occurred but were minimally bothersome and, in the absence of a placebo control, could not be definitively attributed to vaccination. Local reactions, particularly arm pain, were more common among recipients of alum and were usually mild. Of note is the occurrence of itching at the site of injection in six subjects. This appeared to represent an arthus-type response (presumably to the toxoid antigen), rather than type 1 immediate-type hypersensitivity, because the onset was delayed (≥ 1 day after immunization in five of six subjects), inflammation was limited to the site of injection without urticaria or signs of anaphylaxis, and there was no predisposition for recipients of alum-containing formulations (alum is known to drive IgE-mediated reactions). Arthus reactions are common after parenteral vaccine administration and result when immune complexes deposited at the site of injection stimulate vasoactive amines. Since these reactions occur in the presence of pre-existing antibody to the injected antigen, this would explain why itching did not occur after administration of the first dose of vaccine. Nonetheless, a skin biopsy would have been required for definitive diagnosis.

We conclude that *C. difficile* toxoid vaccine is safe and immunogenic in healthy volunteers. Further development of this preparation as a prophylactic vaccine and as an immunostimulatory agent for producing hyperimmune globulin could lead to promising strategies for the prevention and treatment of *C. difficile*-associated diseases.

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