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Doctor
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Director de Nuevas Creaciones (C)
SUPERINTENDENCIA DE INDUSTRIA Y
E. S.

Referencia: Expediente No. 07.109.300
Solicitud de Patente de Invención titulada: "Vacuna de Toxoide de C. Perfringens Alfa"

Solicitante: Schering-Plough Ltd.

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 324 DEL 13 DE JUNIO DE 2012, OFICIO NO. 10219 (EXAMEN DE FONDO)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término de prórroga solicitado en mi memorial 07-109300-00007-0000 de fecha 6 de septiembre de 2012:

1. Objeciones del Examinador

1.1 Excepción a la patentabilidad

Su Despacho menciona que las reivindicaciones 16 a 21 que se refieren a un método para proporcionar la inmunidad pasiva contra múltiples enfermedades de aves de corral no son patentables de acuerdo al artículo 20 d) de la Decisión 486.

Con el fin de atender a la anterior observación, se cancelaron dichas reivindicaciones del

pliego reivindicatorio.

1.2 Reivindicaciones:

Su Despacho menciona en forma resumida lo siguiente con relación al capítulo reivindicatorio:
"el capítulo reivindicatorio no es claro por que no define la estructura química de los antígenos seleccionados para conformar la vacuna.

Se menciona además que no se aceptan definiciones a partir de resultados esperados (efectos esperados de la composición de vacuna), ni definiciones a partir del origen (bacteria, planta o animal) del antígeno que hace parte de la composición...

Las reivindicaciones 13 y 14 tal como se encuentran escritas reclaman la misma materia, el solicitante debe eliminar las repeticiones.....Por último, menciona que el método reclamado en la reivindicación 22 no es claro porque no brinda información suficiente para que una persona versada en la materia pueda...."

1.3 Descripción

Su Despacho establece que la descripción no es clara porque la información que contiene no permite que la persona del oficio normalmente versada en la materia pueda comprender y replicar la invención, específicamente, la invención no proporciona la estructura química exacta de los antígenos que componen las vacunas reclamadas.

1.4 Novedad

Con relación a la novedad su Despacho afirma que los documentos **D1** (WO9323543) y **D2** (US4292307) afectan la novedad de las reivindicaciones 1 a 12.

En forma resumida el examinador afirma lo siguiente:
".....*Las características esenciales de la composición de la reivindicación 1 habían sido divulgadas previamente en D1, el cual divulga una vacuna contra C. perfringens que consta de uno o varios péptidos de 261 a 300 aminoácidos que contienen los epítopes de la toxina*

alfa de C. perfringens tipo A y un adjuvante correspondiente a una emulsión de aceite en agua....En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 11 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Las características esenciales de la composición de la reivindicación 12 habían sido divulgadas previamente en D2, el cual divulga una vacuna contra C. perfringens que consta de toxina de Cl, perfringens tipo A, toxina de Cl perfringens tipo B, toxina de Cl perfringens tipo D, toxina de Cl Oedematiens, toxina de Cl. Septicum.

2. Modificación a una solicitud en trámite:

De conformidad con el artículo 34 de la Decisión 486 que permite al solicitante efectuar modificaciones a su solicitud en cualquier momento del trámite, sin que se presente ampliación de la invención originalmente presentada, me permito presentar un nuevo pliego reivindicatorio como sustituto del originalmente presentado conformado por 13 reivindicaciones.

El examinador podrá notar que el capítulo reivindicatorio que ahora se presenta solo está dirigido a una vacuna para aves, las reivindicaciones 16 a 21 dirigidas a un método para proporcionar la inmunidad pasiva fueron canceladas, así mismo, se cancelaron las reivindicaciones de método para la elaboración de una vacuna de toxoide alfa de C.

Con relación a la observación de su Despacho para las reivindicaciones 13 y 14 que con la nueva numeración corresponden a las reivindicaciones 9 y 10 informamos a su Despacho que dichas reivindicaciones no reclaman la misma materia, por favor nótese que la reivindicación 9 depende de la reivindicación 8 y menciona *"....la vacuna multivalente además comprende uno o más antígenos virales, uno o más antígenos bacterianos y uno o más antígenos parasitarios...."* y

La reivindicación 10 depende de la reivindicación 1 y establece: " *que es una vacuna multivalente que además comprende uno o más antígenos adicionales...".*

Es claro entonces que con el nuevo pliego reivindicatorio se superan las observaciones hechas por su Despacho. Por otro lado, es importante aclararle al examinador que la presente invención no está dirigida hacia una proteína pura y no depende de su específica secuencia de aminoácidos. Por el contrario, la presente invención se dirige a un sobrenadante de toxoide *alfa* de C. *perfringens* tipo A tal como se define en la página 16, líneas 11-24 de la descripción originalmente presentada. Adicionalmente, la proteína alfa de C. *perfringens* tipo A es bien conocida en el estado del arte.

Por lo tanto, las reivindicaciones modificadas que ahora se presentan están claramente definidas por sus características técnicas esenciales y en consecuencia, se concluye que dicho pliego reivindicatorio cumple a cabalidad con el artículo 30 de la Decisión 486.

2. Argumentos a favor de la patentabilidad de la solicitud

2.1 Novedad y Nivel inventivo

La novedad de una solicitud está legalmente definida en el Artículo 16 de la Decisión 486 así: "*una invención se considerará nueva, cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso de la prioridad reconocida...*"

2.2 Nivel inventivo

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando "*si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica*".

En virtud de las anteriores definiciones, consideramos que los documentos D1 y D2 no constituyen documentos del arte previo que afecten la novedad ni el nivel inventivo de la presente solicitud debído principalmente a lo siguiente:

El documento D1 (WO93/23543) Titball *et al* revela fragmentos específicos de la toxina *alfa* de C. *perfringens* que han sido genéticamente modificadas para que esté ausente la actividad de la esfingomielina y/o la fosfolipasa C y el uso de tales péptidos en la preparación de anticuerpos, particularmente anticuerpos monoclonales, y en vacunas (ver resumen). Sin embargo, el documento D1 no enseña las vacunas de la presente invención.

Por lo tanto, dicho documento no puede afectar la patentabilidad de la presente invención. Adicionalmente, no existe nada en dicho documento que conduzca a la persona medianamente experta en la materia a las vacunas de la presente invención.

En particular, aunque Tiball *et al* fue publicada hace 18 años, hasta donde se tiene conocimiento su tecnología revelada no ha sido aún utilizada en una vacuna exitosa. Por lo tanto, la presente invención no es obvia con relación al documento D1 Tiball *et al.*

Con relación al documento D2 (US 4,292,307) Zemlyakova revela lo que ella considera que es una vacuna universal (por favor, ver resumen) que mínimamente comprende toxoides a partir de *Clostridium perfringens* Tipo A, Tipo B, y Tipo D. La presente invención no se refiere a una vacuna que comprende una combinación de toxoides a partir de *Clostridium perfringens* Tipo A, Tipo B, y Tipo D. Por lo tanto, dicho documento no puede de ninguna manera afectar la patentabilidad de la solicitud en estudio.

Adicionalmente, la presente invención no es obvia con relación a D2. El examinador podrá notar que no hay ninguna sugerencia en D2 de que una vacuna que no incluya como mínimo Clostridium perfringens Tipo A, Tipo B, y Tipo D fuera exitosa.

Además, el éxito reclamado reportado por Zemlyakova (ver por ejemplo, líneas 21-35 de la columna 11, y el párrafo puente entre las columnas 11 y 12) le indica a la persona medianamente experta en la materia otro camino diferente de trabajar sobre una vacuna alternativa, dejándolo solo tan diferente a la que enseña Zemlyakova como a las reveladas y reclamadas por los inventores de la solicitud en estudio. Por lo tanto, la presente invención no es obvia con relación a Zemlyakova.

Adicionalmente, el artículo de Stevens *et al* [J. Infect. Diseases 190:767-773 (2004)] es el siguiente artículo a Tiball *et al* [WO93/23543A1]. Como se mencionó anteriormente, Tiball *et al* no enseña las vacunas de la presente invención. Stevens *et al.* emplea el mismo dominio-C de la toxina *alfa* de C. *perfringens* revelado por Tiball *et al.* para diseñar una vacuna para humanos.

En particular, en la página 770, primera columna, Stevens *et al.* señala la dificultad de llegar a una vacuna toxoide al citar a MaClennan [Bacteriol Rev. 26:177-276 (1962)] al concluir:

> "That it was imposible to convert C. perfringens alpha toxin to a toxoid and that any immunizing potency of such preparations resided in residual undenatured toxin. The difficulty of maintainin high antigenicity while totally inactivating alfa-toxin to the extent that such preparation would be safe for human immunization posed insurmountable problems, and thus, a search for alternative vaccine and toxoid preparations became necessary".

En español:

> "..fue imposible convertir la toxina *alfa* de C *perfringens* a un toxoide y que cualquier potencia imnunizadora de tales preparaciones residiera en la toxina no desnaturalizada residual. La dificultad de mantener una alta antigenicidad mientras se inactiva totalmente la toxina alfa en la medida en que tal preparación fuera segura para inmunización humana plantearon insuperables problemas, y por lo tanto, se hace necesario una investigación de una vacuna alternativa y preparaciones de toxoide".

Por lo tanto, la tecnología de Tiball et al., no puede simplemente ser modificada para llegar a la presente invención. En efecto, aunque, Stevens *et al.* también cita un reporte menos desalentador, Stevens et al. escoge emplear un antígeno para la toxina *alfa* de C *perfringens* recombinante truncada en su vacuna.

En consecuencia, los documentos D1 y D2 no proporcionan nada que conduzcan a la persona medianamente experta en la materia a las vacunas de la presente invención. Por lo tanto, la presente invención no es de ninguna manera obvia con respecto a los documentos citados por su Despacho.

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En consecuencia, teniendo en cuenta el nuevo pliego reivindicatorio y los argumentos arriba expuestos, se tiene que la presente solicitud de patente es nueva y posee nivel inventivo.

3. Solicitudes concedidas en otros países para esta misma invención.

A manera informativa, pongo en conocimiento del Examinador que la patente Peruana No. **5426** equivalente a la presente solicitud colombiana ha sido otorgada. Aún cuando entiendo que estas decisiones de ninguna manera obligan al Despacho para decidir de igual forma, considero que por tratarse de una pregunta fáctica la determinación de la existencia de novedad, nivel inventivo y aplicación industrial, las decisiones de otras oficinas de patentes constituyen un antecedente persuasivo en la materia, aún más en éste caso que fue otorgada en Perú que se rige por la misma legislación en materia de patentes a la Colombiana Decisión 486.

En virtud de lo anterior, solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,

ÁLVARO CORREA ORDÓÑEZ
C.C.No.79.143.366 de Usaquén
T.P.20.281

Anexos:

- Capítulo reivindicatorio modificado conformado por 13 reivindicaciones
- Recibo de pago por reivindicación adicional
- Copia del artículo "Immunization with C-Domain..." de Stevens
- Copia del título de patente Peruana No. 5426
- Copia de las reivindicaciones otorgadas en Perú

BM_MGT/BOGDMS-#1248750-V1-P2007_000291-0720.DOC

REIVINDICACIONES

1. Una vacuna para aves que comprende el sobrenadante de toxoide *alfa* de C. *perfringens* tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación total (TCP) de 2 o más; con la condición que solo un antígeno de tipo C. *perfringens* único está presente en dicha vacuna; y

donde dicho sobrenadante de toxoide *alfa* de C. *perfringens* Tipo A comprende un antígeno seleccionado del grupo que consiste en un toxoide alfa de C. *perfringens* tipo A, un fragmento antigénico de un toxoide *alfa* de C. *perfringens* tipo A, un fragmento antigénico inactivo de una toxina *alfa* de C. *perfringens* Tipo A, y cualquier combinación de los anteriores.

2. La vacuna de la reivindicación 1, donde el antígeno se encuentra en una preparación libre de células.

3. La vacuna de la reivindicación 1, donde el ave es un pollo.

4. La vacuna de la reivindicación 1, donde el antígeno es un polipéptido recombinante.

5. La vacuna de la reivindicación 1, que además comprende un adyuvante.

6. La vacuna de la reivindicación 1, donde una emulsión de agua en aceite comprende antígeno.

7. La vacuna de la reivindicación 6, donde la emulsión de agua en aceite se prepare con una fase oleosa al 70% y una fase acuosa al 30%.

8. La vacuna de la reivindicación 1, que es una vacuna multivalente que además comprende una o más de las siguientes toxinas: toxina *beta* de C. *perfringens*, toxina *beta* 2 de C. *perfringens,* enterotoxina de C. *perfringens*, toxina *epsilon* de C. *perfringens*, toxina iota de C. *perfringens*, toxina *kappa* de C. *perfringens*, toxina lambda de C. *perfringens,* toxina theta de C. *perfringens*, toxina *sordellii* de C. hemorrágica, toxina letal de C. *sordellii,* toxina A de C. *difficile*, toxina B de C. *difficile,* toxina alfa de C. *septicum*, toxina alfa de C. *novyi* y toxina *beta* de C. *novyi.*

9. La vacuna de la reivindicación 8, donde la vacuna multivalente además comprende uno o más antígenos virales, uno o más antígenos bacterianos y uno o más antígenos parasitarios;

donde uno o más antígenos virales son de una o más de las siguientes Fuentes: virus de la enfermedad de bursitis infecciosa, virus de la bronquitis infecciosa, reovirus y virus de la enfermedad de Newcastle;

donde uno o más antígenos bacterianos son de una o más de las siguientes fuentes: *E. coli, Salmonella* y *Campylobacter*; y

donde los antígenos parasitarios son de *Eimeria*.

10. La vacuna de la reivindicación 1, que es una vacuna multivalente que además comprende uno o más antígenos adicionales;

donde los antígenos adicionales son de una o más de las siguientes fuentes:

virus de la enfermedad de bursitis infecciosa, virus de la bronquitis infecciosa, reovirus, virus de la enfermedad de Newcastle; *E. coli, Salmonella, Campylobacter*; y *Eimeria*.

11. Una vacuna para aves que consiste esencialmente en el sobrenadante de toxoide *alfa* de C. *perfringens* Tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación Total (TCP) de 2 o más; con la condición que sólo un antígeno de un Tipo C. *perfringens* único está presente en dicha vacuna; y

donde el antígeno toxoide alfa de un Tipo de C. *perfringens* único es un toxoide *alfa* de C. *perfringens* de Tipo A comprendido por dicho sobrenadante de toxoide *alfa* de C. *perfringens* Tipo A.

12. Una vacuna para un pollo que comprende el sobrenadante de toxoide *alfa* de C. *perfringens* Tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación Total (TCP) de 2 o más,

en donde el sobrenadante de toxoide *alfa* de C. *perfringens* Tipo A comprende el antígeno de toxoide *alfa*;

en donde la emulsión de agua en aceite comprende el antígeno con la condición que solo el antígeno toxoide *alfa* de un Tipo de C. *perfringens* único está presente en dicha vacuna.

13. La vacuna de la reivindicación 12 en la cual la emulsión de agua en aceite fue preparada con una fase oleosa al 70% y una fase acuosa al 30%.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-109300- -00008-0000

Fecha: 2012-10-22 08:52:50 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 32

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176089-2

RECIBO OFICIAL DE CAJA : 12 - 109,617
FECHA : OCTUBRE 22 DE 2012

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
BAKER & MCKENZIE	RECIBO DE CAJA			85891	22/08/2012	9,000.00
BAKER & MCKENZIE	RECIBO DE CAJA			85892	22/08/2012	9,000.00
BAKER & MCKENZIE	RECIBO DE CAJA			85893	22/08/2012	9,000.00
BAKER & MCKENZIE	RECIBO DE CAJA			85894	22/08/2012	9,000.00
BAKER & MCKENZIE	RECIBO DE CAJA			85895	22/08/2012	9,000.00
...MAS INFORMACION NO IMP			TOTAL			90,000.00

***** C O N C E P T O *****

CANT.	PENTISTICO	CONCEPTO	TOTAL CONCEPTO
3	50005-01-01 SOLICITUDES	*** REIVINDICACION UNITARIA ADICIONAL	90,000.00
		TOTAL : $	90,000.00

SON: NOVENTA MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No._____

Indecopi

República del Perú

Registro de la Propiedad Industrial

Dirección de Invenciones y Nuevas Tecnologías

TITULO N° 5426

La Dirección de Invenciones y Nuevas Tecnologías del Indecopi certifica que por mandato de la Resolución N° 001332-2009/DIN-INDECOPI de fecha 30 de septiembre de 2009, ha quedado inscrita en el Registro de Patentes de Invención, la siguiente invención:

Denominación	:	VACUNA DE TOXOIDE DE C. PERFRINGENS ALFA
Clasificación	:	A61K 39/08; A61P 31/04; A61P 31/00
Solicitud	:	000396-2006
Fecha de Presentación	:	17 de abril de 2006
Fecha de Prioridad	:	18 de abril de 2005
Titular	:	SCHERING-PLOUGH LTD.
País	:	Suiza
Inventores	:	HUCHAPPA JAYAPPA; KEVIN O'CONNELL
Vigencia	:	17 de abril de 2026

BRUNO MÉRCHOR VALDERRAMA
Director de Invenciones y
Nuevas Tecnologías
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PERÚ | Presidencia del Consejo de Ministros | INDECOPI

PAGO ANUALIDAD
2010 - 2011

PAGO ANUALIDAD
2011 - 2012

PAGO ANUALIDAD
2012 - 2013

TITULO N° 5426

INSTITUTO NACIONAL DE DEFENSA DE LA COMPETENCIA Y DE LA PROTECCIÓN DE LA PROPIEDAD INTELECTUAL
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REIVINDICACIONES

1. Una vacuna para aves que comprende el sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación Total (TCP) de 2 o más; con la condición que sólo un antigeno de Tipo *C. perfringens* único está presente en dicha vacuna; y

donde dicho sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A comprende un antígeno seleccionado del grupo que consiste en un toxoide *alfa* de *C. perfringens* Tipo A, un fragmento antigénico de un toxoide *alfa* de *C. perfringens* Tipo A, un fragmento antigénico inactivo de una toxina *alfa* de *C. perfringens* Tipo A, y cualquier combinación de los anteriores.

2. La vacuna de la reivindicación 1, donde el antígeno se encuentra en una preparación libre de células.

3. La vacuna de la reivindicación 1, donde el ave es un pollo.

4. La vacuna de la reivindicación 1, donde el antígeno es un polipéptido recombinante.

5. La vacuna de la reivindicación 1, que además comprende un adyuvante.

6. La vacuna de la reivindicación 1, donde una emulsión de agua en aceite comprende el antígeno.

7. La vacuna de la reivindicación 6, donde la emulsión de agua en aceite se preparó con una fase oleosa al 70% y una fase acuosa al 30%.

8. La vacuna de la reivindicación 1, que es una vacuna multivalente que además comprende una o más de las siguientes toxinas: toxina *beta* de *C. perfringens*, toxina *beta* 2 de *C. perfringens*, enterotoxina de *C. perfringens*, toxina *épsilon* de *C. perfringens*, toxina *iota* de *C. perfringens*, toxina *kappa* de *C. perfringens*, toxina *lambda* de *C. perfringens*, toxina *theta* de *C. perfringens*,

toxina *sordellii* de *C.* hemorrágica, toxina letal de *C. sordellii*, toxina A de *C. difficile*, toxina B de *C. difficile*, toxina *alfa* de *C. septicum*, toxina *alfa* de *C. novyi* y toxina *beta* de *C. novyi.*

9. La vacuna de la reivindicación 8, donde la vacuna multivalente además comprende uno o más antígenos virales, uno o más antígenos bacterianos y uno o más antígenos parasitarios;

donde uno o más antígenos virales son de una o más de las siguiente fuentes: virus de la enfermedad de bursitis infecciosa, virus de la bronquitis infecciosa, reovirus y virus de la enfermedad de Newcastle;

donde uno o más antígenos bacterianos son de una o más de las siguientes fuentes: *E. coli*, *Salmonella* y *Campylobacter;* y

donde los antígenos parasitarios son de *Eimeria.*

10. La vacuna de la reivindicación 1, que es una vacuna multivalente que además comprende uno o más antígenos adicionales;

donde los antígenos adicionales son de una o más de las siguiente fuentes: virus de la enfermedad de bursitis infecciosa, virus de la bronquitis infecciosa, reovirus, virus de la enfermedad de Newcastle; *E. coli*, *Salmonella, Campylobacter;* y *Eimeria.*

11. Una vacuna para aves que consiste esencialmente en el sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación Total (TCP) de 2 o más; con la condición que sólo un antigeno de un Tipo de *C. perfringens* único está presente en dicha vacuna; y

donde el antígeno toxoide *alfa* de un Tipo de *C. perfringens* único es un toxoide *alfa* de *C. perfringens* de Tipo A comprendido por dicho sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A.

12. Una vacuna para un pollo comprendiendo el sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A que fue concentrado por ultrafiltración y tiene una Potencia de Combinación Total (TCP) de 2 o más,

en donde el sobrenadante de toxoide *alfa* de *C. perfringens* Tipo A comprende el antígeno de toxoide *alfa*;

en donde la emulsión de agua en aceite comprende el antígeno con la condición que sólo el antígeno toxoide *alfa* de un Tipo de *C. perfringens* único está presente en dicha vacuna.

13. La vacuna de la reivindicación 12 en la cual la emulsión de agua en aceite fue preparada con una fase oleosa al 70% y una fase acuosa al 30%.

MAJOR ARTICLE

Immunization with the C-Domain of α-Toxin Prevents Lethal Infection, Localizes Tissue Injury, and Promotes Host Response to Challenge with *Clostridium perfringens*

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Clostridium perfringens **gas gangrene is characterized by rapid tissue destruction, impaired host response, and, often, death. Phospholipase C (α-toxin) is the virulence factor most responsible for these pathologies. The present study investigated the efficacy of active immunization with the C-terminal domain of α-toxin (Cpa247–370) in a murine model of gas gangrene. Primary end points of the study were survival, progression of infection, and tissue perfusion. Secondary end points, which were based on findings of histologic evaluation of tissues, included the extent of tissue destruction and microvascular thrombosis, as well as the magnitude of the tissue inflammatory response. Survival among C-domain–immunized animals was significantly greater than that among sham-immunized control animals. Furthermore, immunization with the C-domain localized the infection and prevented ischemia of the feet. Histopathologic findings demonstrated limited muscle necrosis, reduced microvascular thrombosis, and enhanced granulocytic influx in C-domain–immunized mice. We conclude that immunization with the C-domain of phospholipase C is a viable strategy for the prevention of morbidity and mortality associated with *C. perfringens* gas gangrene.**

Clostridium perfringens gas gangrene is, without a doubt, the most fulminant necrotizing infection that affects humans. The infection can become well established in traumatized tissues in as little as 6–8 h, and the destruction of adjacent healthy muscle can progress several inches per hour, despite appropriate antibiotic coverage. Shock and organ failure are present in 50% of infected patients, and, of these patients, 40% die (reviewed in [1]). Even with modern medical advances and intensive care regimens, the centuries-old practice of radical amputation performed on an emergent basis remains the single best treatment. Furthermore, unlike most other soft tissue infections, clostridial myonecrosis is also remarkable for both the absence of acute inflammatory cells in tissues and the accumulation of leukocytes between fascial planes and within small vessels (leukostasis) near the demarcation between healthy and necrotic tissues [2–4].

Classic studies have identified α-toxin (a phospholipase C; PLC) and θ-toxin (a cholesterol-binding cytolysin) as the principal toxins involved in the pathogenesis of this infection (reviewed in [1]). More recently, elegant genetics-based studies have elucidated the structure/function relationships of both these toxins (reviewed in [4]). Work from our laboratory has demonstrated that PLC contributes to the shock associated with gas gangrene by directly impairing myocardial contractility [5].

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Furthermore, PLC contributes to the rapid destruction of viable tissue by altering inflammatory and endothelial cell functions [6, 7], by stimulating microvascular occlusion [8–10], and by inhibiting the tissue inflammatory response [11].

Williamson and Titball [12] demonstrated that immunization with the C-terminal domain of α-toxin ($Cpa_{247–370}$) induced protection against the lethal effect of the toxin and also ensured the survival of animals challenged with 10 LD_{100} doses of viable *C. perfringens* type A. In the present study, we investigated whether such protection is accompanied by improvements in microvascular function and tissue inflammatory responses.

MATERIALS AND METHODS

PLC C-domain. The recombinant carboxy-terminal domain (i.e., C-domain) used in these studies was produced in *Escherichia coli* as a glutathione-*S*-transferase (GST) fusion with aa 247–370 of α-toxin, as described elsewhere [12, 13]. Therefore, the protein used in our studies was devoid of any other *C. perfringens* proteins. The fusion protein was purified and cleaved from GST by use of factor Xa, and the released C-domain was purified from the GST before use. The isolated C-domain appeared as a single band when analyzed by SDS-PAGE and Coomassie blue staining. Stock C-domain contained 10.2 mg/mL protein that was estimated to be >98% pure.

Animal studies. Animal experiments were approved by the Animal Subjects Subcommittee, Veterans Affairs Medical Center (Boise, ID) and adhered to guidelines of the National Institutes of Health. Female Swiss Webster outbred mice (body weight, 16–18 g) were actively immunized against the PLC C-domain over the course of 9 weeks, by successive intraperitoneal injections of 12.5 μg of C-domain protein in 100 μL of Freund complete adjuvant (for the first immunization) or 100 μL of Freund incomplete adjuvant (for booster immunizations), at 3-week intervals, for a total of 3 doses. Sham-immunized animals received an equal volume of adjuvant alone, according to the same schedule followed for the C-domain–immunized animals. One week after administration of the last booster immunization, 6 animals that were randomly chosen from each immunization group were anesthetized by halothane inhalation, and a blood specimen (500 μL) was obtained from each by means of retroorbital puncture. Serum from these specimens was pooled and was used to verify specific antibody production by Western blot analysis.

The remaining animals in each immunization group were randomly assigned to either a "survival" group ($n = 30$) or a "histopathology" group ($n = 24$). Animals in the survival group were challenged intramuscularly with either 3.75×10^7, 3.75×10^8, or 3.75×10^9 cfu of viable, washed log-phase *C. perfringens* organisms (ATCC 13124), as described elsewhere [14]. After infection, the animals were monitored every 2 h for the first 24 h and then every 12 h, for a total of 7 days. Deaths, as well as signs and symptoms of local and systemic manifestations of gas gangrene, such as scruffy appearance, overall activity level, swelling of the infected limb, blackening of the foot, and use of the infected limb, were recorded. Animals that survived for the entire 7-day period were considered to be survivors and, at the conclusion of the study, were euthanized by administration of halothane anesthesia, followed by cervical dislocation.

Animals in the histopathology group were similarly challenged with either 3.75×10^8 or 3.75×10^9 cfu of *C. perfringens*. At 0, 0.5, 1, 2, 4, and 8 h after infection, 2 animals in each group were killed by means of cervical dislocation. Soft tissues obtained from the infected leg and the uninfected contralateral limb were carefully dissected and fixed in 10% neutral buffered formalin. Tissues were paraffin embedded, sectioned, and stained with hematoxylin-eosin at the University of Idaho Caldwell Veterinary Teaching Center (Caldwell, ID). Tissues were reviewed by one of the investigators (A.E.B.), who was blinded to the sample's origin and who scored the section relative to the extent of tissue necrosis, the magnitude of inflammatory cell infiltration into the tissue, and the degree of vessel occlusion.

RESULTS

Immunization studies. Immunization against the C-domain of *C. perfringens* α-toxin produced a high titer of antibody recognizing both the purified C-domain protein fragment and the intact toxin (figure 1*A*). In contrast, serum obtained from animals that received adjuvant alone (i.e., sham-immunized animals) was devoid of such antibody (figure 1*B*).

Survival studies. At 18 h after infection, survival of animals that were immunized against the α-toxin C-domain and were challenged with 3.75×10^7, 3.75×10^8, or 3.75×10^9 cfu of *C. perfringens* was 90%, 90%, and 80%, respectively, compared with survival of 40%, 0%, and 0% for sham-immunized animals (figure 2, *left*). These survival percentages did not decrease further during the remaining 6 days of the study (not shown).

Sham-immunized animals demonstrated an increase in swelling and loss of function of the infected leg, regardless of the inoculum used to initiate infection (figure 2, *middle*). Similarly, animals immunized against the C-domain demonstrated an increase in swelling and limping after infection. However, these manifestations were localized, transient, and highly dose dependent in this actively immunized group (figure 2, *middle*).

Similarly, sham-immunized control animals that were challenged with the highest inoculum demonstrated a marked blackening of the foot, which was indicative of an irreversible loss of tissue perfusion (figure 2 *[right]* and figure 3). Systemically, all animals in this group had hematuria. However, for animals that were immunized against the C-domain and were similarly challenged, the discoloration of the foot was less intense in color and was transient (figure 2, *right*), and there was no evidence of hematuria. In the groups that received lower

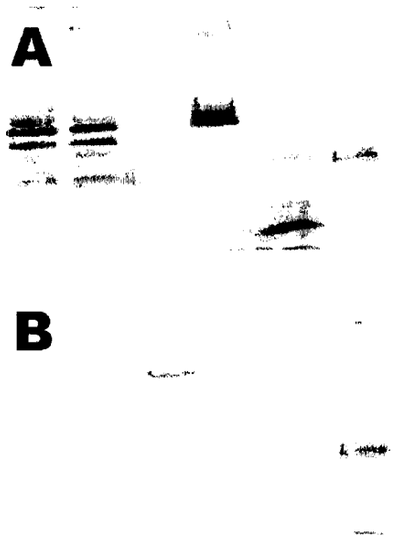

Figure 1. Anti–phospholipase C (PLC) antibody is produced in mice immunized against the C-domain of PLC, as demonstrated by Western blots. *Lanes 1* and *2*, Crude 80% ammonium sulfate precipitate of proteins from log-phase *Clostridium perfringens* (ATCC 13124); *lane 3*, recombinant *C. perfringens* θ-toxin; *lane 4*, recombinant *C. perfringens* PLC; *lane 5*, purified C-domain; *lane 6*, biotinylated molecular weight markers. Blot *A* was developed with serum obtained from mice ($n = 6$) that were actively immunized against the C-domain of *C. perfringens* PLC, as described in Materials and Methods. Blot *B* was developed with serum obtained from mice that received adjuvant alone.

inocula, alterations in perfusion of the foot were significantly less marked (figure 2, *right*).

Histopathologic studies. Sham- or C-domain–immunized animals were challenged with intramuscular injection of either 3.75×10^8 or 3.75×10^9 cfu of washed log-phase *C. perfringens*. Two animals in each group were killed, by means of cervical dislocation, at 0, 0.5, 1, 2, 4, or 8 h after infection, and the tissues of the animals were processed for routine hematoxylin-eosin staining. Few morphologic changes were seen among the tissue samples obtained from animals killed at 0 or 0.5 h after infection in either immunization group, although the inoculum was readily visible (data not shown). By 1 h after infection, focal areas of tissue destruction adjacent to the site of infection were visible, especially in the sham-immunized group (figure 4*A*). A marked inflammatory response was observed in tissue samples obtained from animals immunized against the C-domain (figure 4*B*), whereas it was notably absent in samples obtained from sham-immunized control animals (figure 4*A*). Similarly, by 4 h after infection, animals immunized against the C-domain had more limited tissue destruction, and polymorphonuclear leukocytes were still visible within the infected muscle (compare figure 4*E* and 4*F*). At 8 h after infection, little recognizable tissue architecture was discernable at the infection site in sham-immunized animals, whereas tissues from the C-domain–immunized animals showed little change from the 4-h time point (not shown).

Inspection of the vasculature within and adjacent to the site of infection revealed large occlusive intravascular cellular aggregates in the sham-immunized control animals, at times ≥2 h after infection (figure 4*C*). Vessels from C-domain–immunized animals did not show signs of intravascular aggregate formation (figure 4*D*), except at 4 h after infection with the highest inoculum (not shown).

DISCUSSION

Development of a vaccine for gas gangrene due to *C. perfringens* has been extensively investigated because the infection has a high associated mortality rate, causes extensive morbidity, and often occurs with a high incidence, as exemplified during World Wars I and II. During the Cold War (1950–1970), studies of vaccines were justified on the basis of the likelihood that large numbers of military and civilian casualties would be at risk for gas gangrene after a nuclear holocaust [15]. The present world situation portends another possible scenario of mass casualties of war with extensive injuries conducive to gas gangrene. Thus, for military personnel, the need still exists to develop novel strategies to prevent or attenuate the course of *C. perfringens* gas gangrene. Such strategies could also be safely used either for "at-risk" populations, such as elderly individuals or individuals with diabetes (who may require lower limb surgery as a result of trauma or poor circulation), or for individuals undergoing intestinal surgery. Lastly, a hyperimmunoglobulin could also be developed to treat victims of acute traumatic injury prophylactically and to attenuate the spread of infection in patients with established gas gangrene.

Several studies have demonstrated that active immunization with crude toxoid preparations was protective in experimental infections [15–18]. In addition, the potential efficacy of passive immunization for the prevention of gas gangrene in humans was investigated by British physicians during World War II by immunization of wounded soldiers with gas gangrene antitoxin, a serum preparation obtained from horses immunized with a trivalent toxoid of crude antigens from *C. perfringens*, *C. septicum*, and *C. histolyticum* [19]. Although the investigators did not show that the incidence of gas gangrene was reduced among these wounded soldiers, they did demonstrate that the incubation time for gas gangrene was prolonged from 33 h, among 41 soldiers who did not receive the antitoxin, to 68 h among the 34 soldiers who did receive the antitoxin [19]. These same investigators concluded, albeit somewhat anecdotally, that passive immunization was effective in the treatment of established gas gangrene, so long as antibiotics and surgery were also implemented.

On the basis of early suspicion that α-toxin was the major

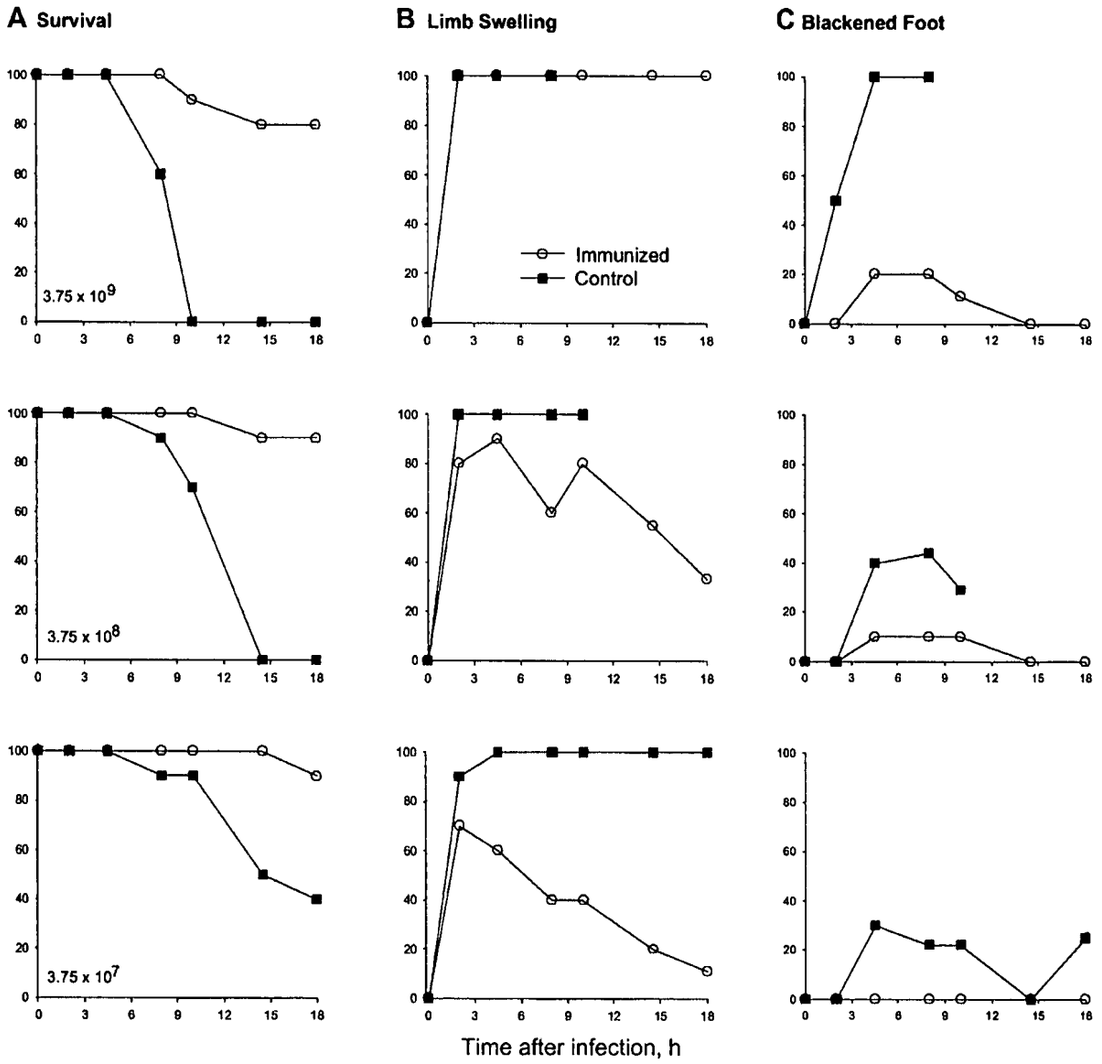

Figure 2. Female Swiss Webster mice were immunized against the C-domain of *Clostridium perfringens* phospholipase C, as described in Materials and Methods. Control animals received adjuvant alone. Animals were challenged with an intramuscular injection, in the right thigh muscle, of either 3.75×10^7, 3.75×10^8, or 3.75×10^9 cfu of washed, log-phase *C. perfringens* (ATCC 13124). Animals were monitored for survival *(A)*, swelling of the infected leg *(B)*, and signs of reduced perfusion (blackening) of the foot distal to the infected site *(C)*. The data reflect the percentage of surviving animals that exhibited these features.

lethal toxin of *C. perfringens*, strategies began using purified α-toxin as the primary immunogen. The results of efficacy studies were varied, causing MacLennan [20] to conclude that it was impossible to convert *C. perfringens* alpha toxin to a toxoid and that any immunizing potency of such preparations resided in residual undenatured toxin. The difficulty of maintaining high antigenicity while totally inactivating α-toxin to the extent that such a preparation would be safe for human immunization posed insurmountable problems, and, thus, a search for alternative vaccines and toxoid preparations became necessary. For example, Ito [21], using highly purified α-toxin, low concentrations of formalin (0.1%), and exogenously added L-lysine, demonstrated high antigenicity, low toxicity, and protection in mice that were immunized with this toxoid and were subsequently challenged with lethal doses of *C. perfringens*.

Although little additional work in this area has occurred for >30 years, modern technology offers some novel strategies, including gene-based alteration of the amino acid sequence of

Figure 3. Severe blackening of the foot was observed in sham-immunized animals challenged with 3.75×10^8 cfu of *Clostridium perfringens*.

α-toxin, truncation of active toxin by a variety of methods, and use of neutralizing monoclonal antibodies directed against α-toxin. In previous studies that used this latter approach, we passively immunized mice with a single dose of neutralizing monoclonal antibody against α-toxin, followed by challenge with a 100% lethal dose of log-phase *C. perfringens* [22]. These studies demonstrated that the early course of gas gangrene could be dramatically improved but that all animals subsequently developed gas gangrene and died. We concluded that a greater degree of protection would require repeated doses of antitoxin because of the shortened half-life and depletion of specific antibody in the face of continued production of α-toxin at the site of infection. Thus, active immunization against α-toxin might provide a nondepletable source of antibody that would be sufficient to protect animals long enough for the acute inflammatory reaction to provide innate resistance.

More recent studies have capitalized on the structure/function attributes of the *C. perfringens* α-toxin. Specifically, α-toxin has an N-terminal domain with sequence similarity to PLC enzymes from other bacteria, as well as a smaller β-sandwich C-terminal domain that is responsible for calcium-dependent membrane binding and hemolysis [23]. Both domains are required for toxicity. Analysis of the α-toxin structure by x-ray crystallography has confirmed the 2-domain structure, with the larger α-helical N-terminal domain containing the active enzymatic site. The C-terminal C2-like domain has strong structural analogy to eukaryotic phospholipid and/or calcium-binding C2 domains, such as those that have been found in intracellular second-messenger proteins and human aracidonate 5-lipoxygenase [24]. The tyrosine residues of the α-toxin C2-like domain interact with the calcium/phosphatidylcholine complex of eukaryotic cell membranes, leading to binding of the toxin to the membrane surface [25] and generation of intracellular messengers, such as diacylglycerol and ceramide [26].

Both of these domains previously have been expressed in *E. coli* and have been purified. Immunization of mice with either of the recombinant domains elicits good antibody responses that react with the toxin [12]. However, only immunization with the C-terminal domain provides protection against a subsequent challenge with α-toxin or protection against gas gangrene in the murine model of disease [12]. The reasons why the C-terminal domain is a protective immunogen are not fully clarified. However, because this domain plays a key role in the binding of the α-toxin to eukaryotic cell membranes [23, 27, 28], it is possible that antibody against this domain blocks the initial membrane-binding event. In support of this possibility, it has been shown that antisera against the C-terminal domain (but not antisera raised against the amino-terminal domain) are able to inhibit the hemolytic activity of the toxin [12]. The epitopes in the C-terminal domain that are responsible for the induction of neutralizing antibody have not yet been identified. However, previous studies have shown that immunization with the recombinant C-terminal domain, derived from *C. perfringens* strain ATCC 13124 (a type strain), provides protection against the α-toxin produced by *C. perfringens* strain CER89L43, which was isolated from a diseased calf [29]. Of importance, the *C. perfringens* strain CER89L43 and the ATCC 13124 α-toxins show significant sequence diversity, including diversity in the C-terminal region. This finding suggests that the C-terminal domain could serve as a vaccine that is protective against α-toxin from a wide range of *C. perfringens* isolates.

Using genetic techniques to develop a truncated α-toxin encompassing aa 247–370 ($Cpa_{247-370}$), Williamson and Titball demonstrated that this protein had no toxicity and that antibody directed against this protein neutralized both the phospholipase and hemolytic activities of PLC [12]. Furthermore, Williamson and Titball [12] demonstrated that active immunization with 3 doses protected mice against lethal challenge with wild-type *C. perfringens*.

Results from the present study confirm that active immunization of mice with the C-domain of α-toxin ($Cpa_{247-370}$) provided protection to 80%–90% of mice challenged with 10–100 lethal doses of washed, log-phase *C. perfringens*. In addition, active immunization greatly altered the early histopathologic findings of infection, whereas sham-immunized mice demonstrated an absence of leukocytic infiltration, accumulation of neutrophil-platelet aggregates intravascularly, and rapid progression to typical gas gangrene of the entire extremity. Of interest, at the site of infection, vaccinated animals had evidence of neutrophil influx to the site of inoculum as early as 1 h after infection. In addition, blood vessels adjacent to the inoculum were devoid of leukocyte-platelet aggregates. At later time points, these histopathologic differences were striking.

The general appearance of the wound reflected the histopathologic differences in that infection in vaccinated animals remained localized to the site of inoculation, probably as a result

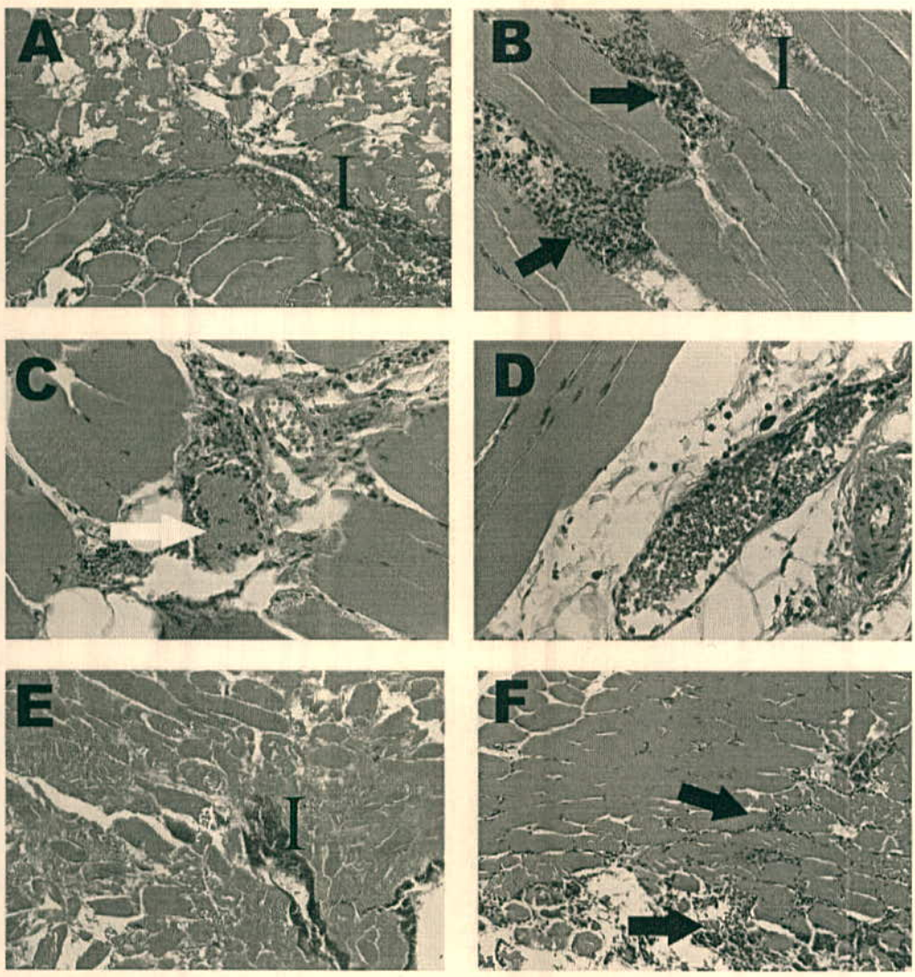

Figure 4. Tissue inflammatory response to intramuscular infection with *Clostridium perfringens*. Animals were actively immunized against the phospholipase C C-domain, as described in Materials and Methods (*B, D,* and *F*). Control animals received adjuvant alone (*A, C,* and *E*). At 1 h (*A* and *B*), 2 h (*C* and *D*), or 4 h (*E* and *F*) after infection with 3.75×10^8 cfu of *C. perfringens*, tissue from the infected site was harvested and processed for routine hematoxylin-eosin staining. At 1 h after infection, extensive necrosis of tissue was apparent in the sham-immunized animals, a line of demarcation between healthy and necrotic tissue was visible, and infiltrating polymorphonuclear leukocytes (PMNL) were absent *(A)*. In contrast, muscle had a relatively good appearance in the C-domain–immunized animals *(B)*, and PMNL influx was clearly visible *(solid arrows)*. Occlusive intravascular aggregates appeared in the vessels adjacent to the site of infection in tissues obtained from sham-immunized animals *(C; white arrow)*, whereas vessels in the C-domain–immunized animals remained patent *(D)*. By 4 h after infection, muscle destruction was more widespread in the sham-immunized animals, and the tissue inflammatory response remained absent *(E)*. In contrast, the skeletal muscle in the C-domain–immunized animals showed significantly less necrosis, and the PMNL influx was more pronounced than that noted for the sham-immunized animals *(F)*. Solid arrows denote infiltrating PMNL. I, Bacterial inoculum.

of containment by the acute inflammatory reaction, just as *Staphylococcus aureus* infection is contained by neutrophils leading to a localized abscess and not a progressive necrotizing lesion. Although some small aggregates were observed in the blood vessels of vaccinated animals, these aggregates were dramatically less apparent than those observed in sham-immunized control animals. That these small aggregates did not lead to permanent compromise of the microvasculature is confirmed by our observations that early cyanosis of the foot of the affected leg was transient and resolved within hours. In sharp contrast, early cyanosis of the foot in sham-immunized control animals gradually progressed to cold, blackened feet.

Taken together, results from the present study demonstrate excellent efficacy of the truncated C-domain of α-toxin as an

immunogen for vaccination. In addition, these findings support our hypothesis that α-toxin–induced dysregulation of the immune response of the host fails to contain *C. perfringens* locally. Furthermore, α-toxin–induced activation of neutrophils, platelets, and endothelial cells results in the formation of occlusive intravascular aggregation, such that perfusion decreases dramatically and irreversibly in adjacent tissues [9, 10]. To the host, this represents accelerated ischemic necrosis and loss of limb. To the anaerobic bacterium, *C. perfringens*, this pathogenic scenario ensures an ideal hypoxic environment.

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