た。

F. 健康危険情報
なし

G. 論文発表
なし。

H. 知的財産権の出願・登録状況
なし。

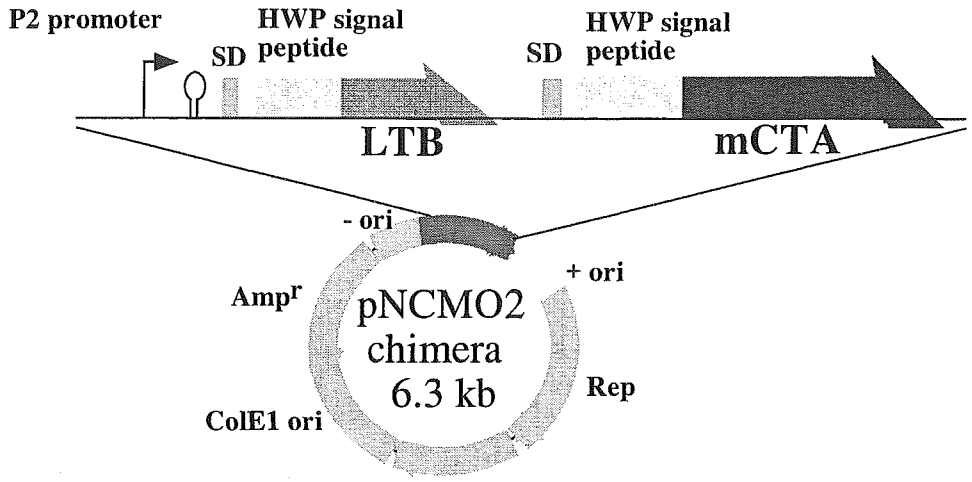
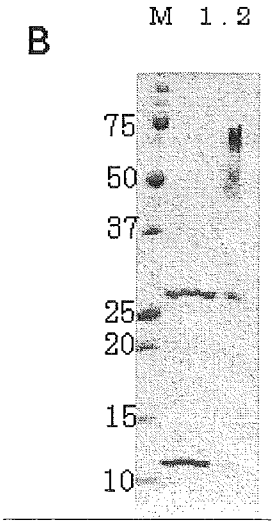


図1. pNCMO2-chimer プラスミド

A

Sample	frac.No.	conc. (mg/ml)	Endotoxin (EU/ml)	chimera (mg)
1	1-37	0.5	50	18.5
2	55-79	0.5	200	12.5
3	1-36	0.6	50	21.6
4	46-81	0.6	200	21.6
5	1-81	0.5	200	40.5
6	1-22	0.5	200	11
total				125.7

B



1 : reducing
2 : nonreducing

図2. 3Lジャーファーメンターでの試作品の生産量とエンドトキシン濃度（A）及びSDS－PEGE分析

厚生科学研究費補助金（新興・再興感染症研究事業）
分担報告書

ペプチド型アジュバントの開発

分担研究者　竹田美文　（株）シネ・サイエンス研究所・所長
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研究要旨：　CTのペプチド型アジュバント開発に向け、CTのアジュバント活性を担う部位を同定するため、nCT, mCT, nCTA, mCTA およびCTBを精製した。これらをアジュバントとし、OVAを抗原としてマウス経鼻投与実験を行ったところ、nCTあるいはnCTAをアジュバントとして加えたグループにのみ強い免疫が惹起された。nCTおよびmCTを用いてGM1-ELISAを行ったところ、両分子のGM1への結合性は同等であった。またNative-PAGEによりnCT, mCT, nCTA, mCTA およびCTBの移動度を比較したところ、nCTに比べmCTが、またmCTAに比べmCTAが、若干遅れて泳動されるものの、ほぼ同じ移動度を示す事がわかった。これらの結果から、nCTおよびnCTAに比べmCTおよびmCTAのアジュバント活性が低いのは、GM1への結合親和性の低下や立体構造の崩壊によるものではなく、毒性であるADP-リボース転移酵素活性の消失によることが示唆された。

A. 研究目的：　コレラ毒素（CT）が強い粘膜アジュバント活性を有することはよく知られているが、そのメカニズムは不明である。CTBのみでは弱いアジュバント活性がホロ毒素の微量の混入により飛躍的に上昇することや、１アミノ酸置換により毒性を減弱させた変異CTでもアジュバント活性を有することから、本研究ではCTAの構造的特徴がアジュバント活性を担うという仮説に基づき、CT分子のアジュバント活性を担う部位の同定を試み、さらにその部分のみの合成ペプチドによる新規粘膜アジュバント開発に向けた基礎的データを得ることを目的とする。このようなペプチド型のアジュバントは、CT特有の下痢毒性を持たないため安全で、かつ人工合成できるため安価で供給することが可能であると考えられ、本邦のみならず開発途上国における感染症予防に多大な貢献が期待できる。

B. 方法：　野生型ホロCT（nCT）、Aサブユニット（nCTA）およびBサブユニット（CTB）、112番目のグルタミン酸をリジンに変異させ無毒化した変異ホロCT（mCT）およびそのAサブユニット（mCTA）をHis-Tagベクターで発現させ、Ni-カラムで精製し、さらにHis-Tagを切断して再度カラム精製し、PolymixinBカラムによりエンドトキシンを除去し最終サンプルとした。

上記各CT分子を、SDS-PAGEおよびnative PAGEで泳動し、泳動パターンを確認・比較した。

nCTとmCTのGM1への結合性の差を調べるため、GM1-ELISAを行った。すなわち、nCT、mCT、CTB、そしてコントロールとしてコレラ菌由来のCT、大腸菌組換え体由来のCTおよび*Brevibacillus chosinensis*組換え体由来のCTを用い、96穴プレートを2 μg /mlのGM1でコートし、各CT分子を100 μg/mlから4倍希釈列を0.38 ng/mlまで作製しウエルにアプライし4°Cで O/Nインキュベートした。洗浄後、ウサギ抗CT抗血清を100倍希釈でアプライし、２時間インキュベートした。洗浄後、HRP-抗ウサギIgGを1000倍希釈でアプライし、1時間インキュベートした。洗浄後、ABTS-H_2O_2により発色反応を行ない、A405を測定した。

各CT分子（10 μg、nCTのみ1 μg）を卵白アルブミン(OVA、100 μg)とともにマウス(C57BL/6、雌、5週齢）に1週間間隔で3回経鼻投与し、最終投与から1週間目に血液、便、鼻洗浄液を採取し、OVAに対する血清IgG、鼻洗浄液IgAおよび糞便IgAをELISAにより測定した。

（倫理面への配慮）
動物実験は国立国際医療センター研究所の動物取扱規程に準拠して行われた。

C. 結果：　nCTおよびmCTのホロ毒素およびサブユニット分子のアクリルアミドゲル電気泳動の結果、SDS-PAGE（還元条件）ではnCTとmCT、あるいはnCTAとmCTAには全く違いは見られなかった。一方、native-PAGEでは、nCTに比べmCTが、またmCTAに比べmCTAが、それぞれ若干遅れて泳動されるものの、ほぼ同じ移動度を示す事がわかった。

nCTとmCTのGM1に対する結合能をGM1-ELISA

により確認したところ、図１に示すように、これら両者の間に有意な差は見られなかった。また、コレラ菌、大腸菌、*B. chosinensis* 由来のCTもほぼ同じGM1結合能を示した。

OVA 抗原に対するアジュバント活性をマウス経鼻投与で比較したところ、図２に示すように、血清IgG、鼻洗浄液IgAともにnCTあるいはnCTAを加えたグループのみが高い抗 OVA 抗体価を示した。糞便 IgA はいずれも検出限界以下であった（データは示さない）。

D. 考察： 本研究において現在まで、nCTとmCTの各サブユニットの OVA 抗原を用いたマウス経鼻投与によるアジュバント活性は、nCT およびnCTAが高い活性を示し、mCT、mCTA、CTBの活性は低かったこと、また tetanus toxoid (TT) を抗原とした同様の実験ではnCTAのアジュバント活性はCTBと同程度まで低下したことを報告し、OVAがキャリアータンパクとしてnCTAの免疫細胞への供給を促進した可能性を示唆した。またnCTのCHO細胞毒性(CD50)は0.2 ng/mlで、nCTAの毒性はその$1/10^4$程度であり、またmCT、mCTA、CTBは無毒であったことを示し、マウス経鼻投与による粘膜アジュバント活性は、CTの毒性の本体であるADP-リボース転移酵素活性を効率よく免疫担当細胞にデリバーし発揮する事に帰する可能性を示唆した。

今回得られたマウス経鼻投与による結果では、やはりmCTAのみならずmCTも、高いアジュバント活性を示さなかった。上述の結果と合わせて考えると、CTの粘膜アジュバント活性は、毒性の本体であるADP-リボース転移酵素活性に大きく依存していることが強く示唆される。この結果は本研究と同じ変異コレラ毒素を用いたYamamotoら（J Exp Med 185:1203-1210, 1997）の結果を再現していないが、現在はRappuoliら（Immunol Today 20: 493-500, 1999）の総説に見られるように、CT のアジュバント活性は、BサブユニットのGM1への結合、ホロ毒素の構造、ADP-リボース転移酵素活性の３つの要因が複合的に寄与していることが判明してきた。本研究で目指しているペプチド型のアジュバントは、当然毒性であるADP-リボース転移酵素活性を持たせる事は出来ない。従って新規ペプチド型アジュバントを開発するには、CTのアジュバント活性発揮のメカニズムを解明し、その上でコストとベネフィットのバランスを考慮し進めて行く必要がある。

現在、この結果の再現性を、mCTA/LTB（Kweon et al. J Infect Dis 2002 186:1261-1269）を加えマウス経鼻投与系で追試するとともに、各CTサンプルが免疫細胞に与える影響を、マイクロアレイアッセイを用い詳細に検討している。

E. 結論： CTのアジュバント活性を担う部位を同定するため、nCT, mCT, nCTA, mCTA およびCTBを精製し、OVAを抗原としてこれらのアジュバント活性をマウス経鼻投与で追試したところ、やはりADPリボース転移活性のあるnCTとnCTAにのみ強いアジュバント活性が認められた。nCTとmCTのGM1への結合性を調べたところ、ほぼ同等である事がわかった。またNative-PAGE上での挙動を比較したところ、nCTとmCT、あるいはnCTAとmCTAは、mutantの方が若干遅れるものの、ほぼ同じ移動度を示す事がわかった。これらの結果から、nCT およびnCTAに比したmCTおよびmCTAのアジュバント活性の低下は、GM1への結合親和性の低下や立体構造の崩壊によるものではなく、毒性であるADP-リボース転移酵素活性の消失によるところが大きいと示唆された。

F. 健康危険情報

特記すべきことなし。

G. 研究発表

１．論文発表

なし

２．学会発表

1）清水　健、濱端　崇、竹田美文、林　英生、太田敏子。腸管出血性大腸菌が産生する志賀様毒素の認識性の解析。第78回日本細菌学会総会、2005年４月、東京。

2）清水　健、濱端　崇、川上怜美、林　英生、太田敏子。志賀様毒素の受容体認識決定領域の解析。第52回毒素シンポジウム、2005年7月、仙台。

H. 知的財産権の出願・登録状況（予定を含む）

１．特許取得　　なし

２．実用新案登録　なし

３．その他　　なし

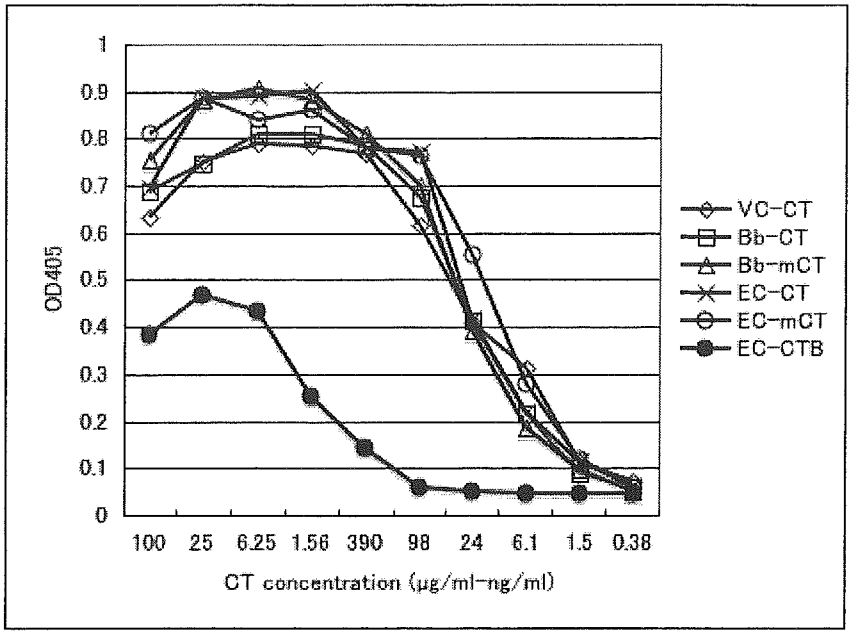

図１．各 CT 分子の GM1 に対する結合性。それぞれ *V. cholerae* (VC-)、*B. chosinensis* (Bb)、*E. coli* (EC) を宿主として発現・精製した野生型 CT (CT)、mutant CT (mCT) および CTB を、100 μg/ml から 0.38 ng/ml まで段階希釈し、GM1 との結合性を ELISA で測定した。縦軸は 405 nm の吸光度。一次抗体には抗 CT 抗血清（ポリクローナル）を用いたため、CTB の吸光度は低くなっている。

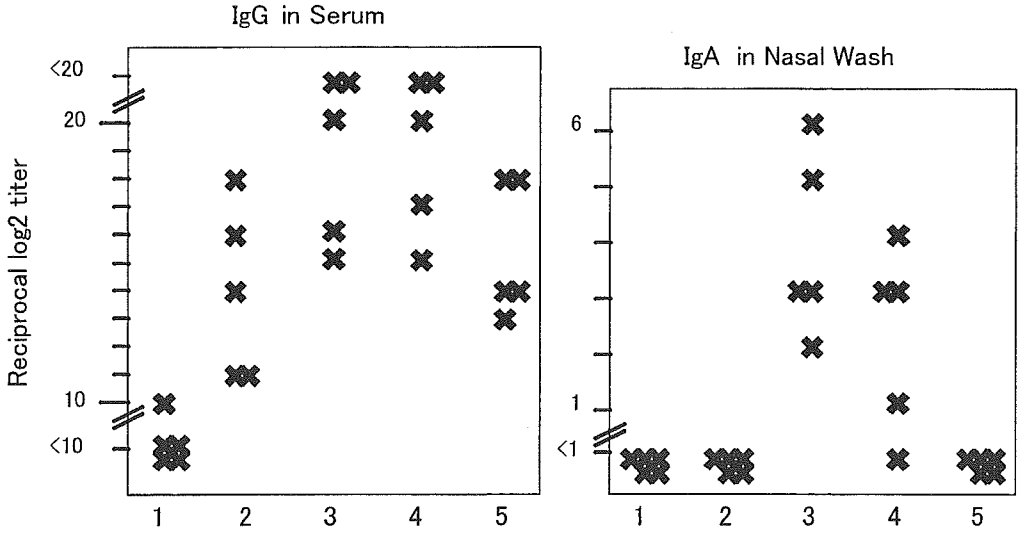

図２．マウス経鼻投与による各 CT 分子のアジュバント活性。血清中の IgG (IgG in Serum) および鼻洗浄液中の IgA を ELISA で測定し、各個体の力価データを×でプロットした。両グラフとも 1. OVA のみ (100 μg)、2. OVA + CTB (10 μg)、3. OVA + nCT (1 μg)、4. OVA + CTB + nCTA (10 μg)、5. OVA + CTB + mCTA (10 μg)。

厚生労働科学研究費補助金（新興・再興感染症研究事業）
分担研究報告書

TLRをターゲットとした新規粘膜アジュバント開発

分担研究者　竹田　潔　九州大学生体防御医学研究所教授

研究要旨:アジュバント受容体であるTLRを介した自然免疫系の活性化の制御機構を解析した。特に、腸管粘膜免疫系の自然免疫系の活性制御機構を解析するため、正常マウスと慢性大腸炎を発症するIL-10ノックアウトマウスの大腸粘膜固有層マクロファージ間で遺伝子発現の差をDNAマイクロアレイで解析した。その結果、IκBファミリーに属するIκBNSが正常大腸粘膜固有層マクロファージに特異的に発現していることを見出した。IκBNSの生理機能を解析するためノックアウトマウスを作製したところ、IκBNSノックアウトマウス由来のマクロファージでは、TLR刺激により誘導される遺伝子の中で、IL-6などのNF-κB依存性に3時間以降に遅れて誘導されてくる遺伝子の発現が有意に上昇していた。またTLR刺激によるNF-κBの活性が遷延化し、刺激後3時間でもNF-kBの活性が残存していた。さらに、dextran sodium sulfateの経口投与による腸管炎症に対する感受性も極めて高くなっていた。以上の結果から、核に発現するIκB分子IκBNSが、NF-κBの活性を抑制することにより自然免疫系の活性を制御し、個体レベルで腸管炎症抑制に関与していることが明らかになった。

A. 研究目的

自然免疫系が、Toll-like receptor (TLR) ファミリーメンバーにより、病原微生物の生体内侵入を感知し、自然免疫系の活性化のみならず抗原特異的な獲得免疫系の活性化をも誘導することが、明らかになってきた。さらに、TLRは、CpG DNAやcomplete Freund's adjuvant (CFA)などアジュバントを認識することも明らかになっている。しかし、TLRを介した自然免疫系の活性化は、その制御機構が破綻すると、慢性炎症性腸疾患などの慢性炎症を引き起こすことも明らかになっている。そこで新規のより効果的なアジュバントの開発に向けた基礎的基盤を提供するため、自然免疫系の活性制御機構を解析し、新規アジュバントによる自然免疫系のより効果的な活性制御技術の開発に資することを、本研究の目的とする。

B. 研究方法

自然免疫系の活性制御機構を解析するためのモデルとして、自然免疫系の活性制御機構の破綻により慢性大腸炎を発症する、自然免疫系特異的Stat3欠損マウス、IL-10ノックアウトマウスを用いて解析を行った。

正常マウスの大腸の粘膜固有層には少数のマクロファージや樹状細胞が存在している。これらの細胞群の単離法を確立し、高純度のマクロファージや樹状細胞を培養できるようになった。正常マウスの大腸の粘膜固有層由来の細胞は、IL-12などの炎症性サイトカインを産生しない。一方、慢性腸炎を発症するIL-10ノックアウトマウスやStat3変異マウスの大腸の粘膜固有層では、たとえ慢性腸炎を発症する前の若いマウスでも、マクロファージや樹状細胞の数が極めて増加しており、さらにこれらの細胞はIL-12などの炎症性サイトカインを産生することを明らかにした。そこで、正常マウスの細胞がサイトカイン産生を示さない分子機構を、正常マウスとIL-10ノックアウトマウスの細胞間で遺伝子発現の差をDNA microarrayで解析し、核に発現するIκB分子IκBNSが、正常マウスの細胞に選択的に発現していることを見いだした。IκBNSをマクロアージ系細胞株に発現させると、LPS刺激によるTNF-α産生は抑制しないが、IL-6産生を抑制することを見いだした。そこで、IκBNSの生理機能を明らかにし、この個体レベルでの役割を明らかにするため、ノックアウトマウスを作製し、その表現型を解析した。

（倫理面への配慮）

本研究は実験動物を用いたものであるが、実験動

物の飼育は、空調設備、照明の時間制御の整ったSPF環境化で週に1回の床敷交換、餌水分補給を専門職員に委託し、行っている。また、毎年秋に動物慰霊祭を行っている。また実験に当たっては、麻酔操作を行い、極力苦痛の軽減を行うよう配慮している。

C. 研究結果

IκBNSノックアウトマウスは正常に出生し、外見上異常は認めなかった。IκBNSノックアウトマウスより腹腔マクロファージを単離、あるいは骨髄由来樹状細胞を分化させ、TLR刺激によるTNF-α, IL-6, IL-12産生を解析した。その結果、IκBNSノックアウトマウスでは、TNF-α産生に変化はないが、IL-6, IL-12産生が有意に亢進していた。次にこれら遺伝子のmRNAの発現誘導をreal time RT-PCR法で解析した。TNF-α mRNAの誘導は刺激後1時間以内に認められるが、この誘導パターンには正常マウスとIκBNSノックアウトマウス間で変化はなかった。IL-6 mRNAは刺激後3時間から誘導がみられるが、3時間までは両マウス間で差はなかった。正常マウスでは3時間以降mRNAの発現量は低下するが、IκBNSノックアウトマウスではmRNA量は高いままであった。他の遺伝子にも刺激後1時間以内の早期に誘導されてくる遺伝子(IL-1beta, IL-12p19など)と遅れて時間以後に誘導される遺伝子(IL-12p40, IL-18など)があるが、早期に誘導される遺伝子群の発現誘導パターンは両マウス間で差がないが、遅れて誘導されてくる遺伝子群は5時間以降IκBNSノックアウトマウスで有意に発現が高かった。次に、TLR刺激によるまたTLR刺激によるNF-κBの活性化をゲルシフト法、およびNF-κB p65の細胞内局在により解析した。その結果、IκBNSノックアウトマウスではNF-κBの活性が遷延化し、刺激後3時間でもまだNF-κBの活性が残存していることが明らかになった。次に、TNF-α, IL-6プロモーターへのNF-κB p65の会合をクロマチン免疫沈降法で解析した。TNF-aプロモーターへのp65の会合は刺激後1時間をピークに観察され、これは正常マウスとIκBNSノックアウトマウスの間で差は認められなかった。一方IL-6プロモーターへのp65の会合は刺激後3時間をピークに正常マウスでは認められた。IκBNSノックアウトマウスでも3時間までは正常マウスと同様にp65の会合が誘導されたが、3時間以降正常マウスではp65の会合が減少していくのに対し、IκBNSノックアウトマウスでは持続したままであった。これらの結果から、IκBNSは刺激後遅れて誘導されてくる遺伝子のプロモーターにおいて選択的にNF-κBの活性を抑制することにより、遺伝子発現を抑制していることが明らかになった。さらに個体レベルでも、LPS投与によるエンドトキシンショックに対する感受性が高くなり、またdextran sodium sulfateの経口投与による腸管炎症に対する感受性も極めて高くなっていた。

D. 考察

以上の結果から、核に発現するIκBNSが、自然免疫系の細胞においてNF-κBの活性を抑制することにより、あるサブセットの遺伝子発現を抑制し、個体レベルで炎症抑制に関与していることが明らかになった。今後も、自然免疫系の活性制御機構を解析し、その制御技術基盤を確立し、有効なアジュバントの創出に寄与したい。

E. 結論

大腸の粘膜固有層に局在する自然免疫系細胞は、TLR刺激に応答しない。そしてその不応答機構の破綻が慢性炎症性腸疾患の発症のトリガーとなりうる。正常では、核に発現するIκB分子IκBNSが選択的にTLR刺激依存性のサイトカイン産生を負に制御し、過剰な炎症の誘導を抑制している。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

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研究成果の刊行物・別刷

A Novel Neurotoxoid Vaccine Prevents Mucosal Botulism[1]

Ryoki Kobayashi,* Tomoko Kohda,† Kosuke Kataoka,* Hideshi Ihara,† Shunji Kozaki,† David W. Pascual,‡ Herman F. Staats,§ Hiroshi Kiyono,*¶ Jerry R. McGhee,* and Kohtaro Fujihashi[2]*

The threat posed by botulism, classically a food- and waterborne disease with a high morbidity and mortality, has increased exponentially in an age of bioterrorism. Because botulinum neurotoxin (BoNT) could be easily disseminated by terrorists using an aerosol or could be used to contaminate the food or water supply, the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases has classified it as a category A agent. Although clearly the development of a safe and effective mucosal vaccine against this toxin should be a high priority, essentially no studies to date have assessed mucosal immune responses to this disease. To bridge this gap in our knowledge, we immunized mice weekly for 4 wk with nasal doses of BoNT type A toxoid and a mutant of cholera toxin termed E112K. We found elevated levels of BoNT-specific IgG Abs in plasma and of secretory IgA Abs in external secretions (nasal washes, saliva, and fecal extracts). When mice given nasal BoNT vaccine were challenged with 4×10^3 LD_{50} of BoNT type A (BoNT/A) via the i.p. route, complete protection was seen, while naive mice given the same dosage died within 2 h. To further confirm the efficacy of this nasal BoNT vaccine, an oral LD_{50} was determined. When mice were given an oral challenge of 5 μg ($2 \times$ oral LD_{50}) of progenitor BoNT/A, all immunized mice survived beyond 5 days, while nonimmunized mice did not. The fecal extract samples from nasally vaccinated mice were found to contain neutralizing secretory IgA Abs. Taken together, these results show that nasal BoNT/A vaccine effectively prevents mucosal BoNT intoxication. *The Journal of Immunology,* **2005, 174: 2190–2195.**

It is now clear that parenteral immunization usually fails to elicit protective mucosal immune responses (1). Various alternate routes of Ag-vaccine delivery have been suggested by researchers, but none has shown more promise for the induction of Ag-specific Ab responses in the upper mucosal compartments (e.g., the respiratory tract) than nasal administration or in the gastrointestinal (GI)[3] tract than oral administration (2–7). Our own previous studies have shown that gut-associated lymphoreticular tissue-directed oral immunization effectively induces Ag-specific secretory IgA (S-IgA) Ab responses in the small intestinal lamina propria (iLP) and the submandibular glands, but not in the lung or the lamina propria of the nasal or reproductive tracts (5–9). In most current nasal immunization studies, the vaccine is instilled into each nostril (usually ~5 μl/nostril), and normal inhalation results in the effective delivery of the vaccine, presumably into nasopharyngeal-associated lymphoreticular tissues (NALT). Such NALT-mediated nasal immunization has been shown to elicit significant immunity in the respiratory, GI, and reproductive tracts as well as in the nasal and oral cavities. These findings suggest that the nasal route of Ag delivery may possess potential advantages in the induction of mucosal immunity.

Native cholera toxin (CT) and mutants of CT (mCTs) are effective mucosal adjuvants and have been widely used for nasal immunization with protein Ags, bacterial components, viruses, or virus-related peptides for the induction of protective immunity associated with S-IgA and plasma IgG Ab responses (4, 10–12). Nasal immunization with the weakly immunogenic OVA along with mCT as adjuvant resulted in S-IgA anti-OVA Ab responses in various mucosal external secretions (4). Furthermore, mice nasally immunized with pneumococcal surface protein A Ag plus mCT revealed pneumococcal surface protein A-specific S-IgA Ab responses associated with effective protection against capsular serotype 3 *Streptococcus pneumoniae* A66 (10). These Ag-specific S-IgA Ab responses were associated with polarized Th2-type responses in cervical lymph nodes (4, 10). Nasal immunization with diphtheria toxoid plus mCT E112K has also been shown to induce protective immunity to the diphtheria exotoxin (11). Furthermore, our recent studies showed that young adult as well as aged mice given a nasal vaccine of tetanus toxoid along with a chimera of mCT-A E112K and heat labile toxin (LT) B subunit were protected when mice were challenged via the systemic route with tetanus toxin (12, 13).

Clostridium botulinum is an anaerobic bacterium that produces a powerful exotoxin termed botulinum neurotoxin (BoNT), which

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Received for publication August 31, 2004. Accepted for publication November 8, 2004.

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[1] This research was supported by National Institutes of Health Grants DE 12242, AI 43197, AI 18958, AI 35932, AI56286, DC 04976, and DK 44240, as well as grants-in-aid from CREST of Japan Science and Technology Corporation, the Ministry of Education, Science and Sports, and the Ministry of Health and Labor, Japan.

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[3] Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; BoNT, botulinum neurotoxin; BoNToxoid, botulinum neurotoxoid; CT, cholera toxin; iLP, intestinal lamina propria; LT, labile toxin; mCT, mutant of CT; NALT, nasopharyngeal-associated lymphoreticular tissue; NP, nasal passage; S-IgA, secretory IgA.

0022-1767/05/$02.00

induces flaccid paralysis (14–17). The BoNTs, serologically divided into seven immunologically different types (A through G), are released from the bacterium as a single polypeptide chain (14–17). Among these seven serotypes, types A, B, and E are typically associated with botulism in humans, and type C has been the most common cause of disease in domestic animals. However, both C and D induce botulism in cows (15–18). As with foodborne botulism, type A is responsible for 60%, type B for 30%, and type E for 10% (18) of cases in humans. It should be emphasized that bioterrorists could potentially use any or all seven serotypes. The molecular mass of each exotoxin type is ~150 kDa (14–17). Each consists of two polypeptide chains, an L chain (50-kDa) and an H (100-kDa) chain. The main targets of BoNT are the peripheral cholinergic nerve endings and particularly the cholinergic neuromuscular junction. BoNT inhibits exocytosis, inducing the flaccid paralysis characteristic of botulism and eventually leading to death (19–25).

The bacterium *C. botulinum* and its derived BoNTs enter the body most commonly through the GI tract via tainted food. In the case of a bioterrorist event, the BoNT would most likely be disseminated by airborne or possibly waterborne routes. BoNTs are classified as one of the six highest risk agents for bioterrorism (category A agents) by the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases. Any chance of surviving this extremely potent and lethal toxin rests upon prolonged intensive care. The current pentavalent botulinum vaccine (serotypes A-E) is used for at-risk populations such as scientists researching BoNTs, health care providers dealing with their clinical manifestations, and the military (26). Furthermore, the current pentavalent vaccine is administered via the parenteral route and induces prolonged pain and swelling at the site of injection (26) (H. F. Staats, unpublished results) To minimize these side effects, several research groups have attempted to develop BoNT vaccines using recombinant C-terminal half of the H chain of BoNT peptide or synthetic epitopes (27–32). Furthermore, a Venezuelan equine encephalomyelitis virus replication vector system provided effective protection against BoNT intoxication (33). However, given the nature of botulinum intoxication, it is essential to devise more effective mucosal vaccines to prevent the intoxication at both mucosal and systemic tissue compartments.

In this study, we have examined whether a nasal vaccine consisting of botulinum neurotoxoid type A (BoNToxoid/A) and mCT E112K as adjuvant would effectively induce protective immunity to BoNT/A challenge. Our results indicate that this nasal vaccine induced BoNT-specific Ab responses in both mucosal and systemic lymphoid tissues. Furthermore, the nasal vaccine prevented botulinum intoxication following either mucosal or parenteral challenge.

Materials and Methods

Mice

Young adult (6–8 wk old) C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute). Upon arrival, all mice were immediately transferred to microisolators and maintained in horizontal laminar flow cabinets and provided sterile food and water ad libitum. Experiments were performed using young adult C57BL/6 mice between 6 and 8 wk of age. The health of the mice was tested semiannually, and mice of all ages used in these experiments were free of bacterial and viral pathogens.

Preparation of BoNToxoid/A

The BoNT/A from *C. botulinum* type A 62 was used for toxoid preparation. The BoNT/A was purified using previously described methods (15). Briefly, purified BoNT/A was detoxified at 30°C by dialyzing against 0.2% Formalin in 0.1 M phosphate buffer (0.2 mg/ml, type A toxin containing 3.5, 10^6 LD_{50}/ml). The detoxification proceeded at 30°C. The formalized toxin was sampled at intervals for mouse inoculation until it became completely nontoxic. At each interval, two mice were i.p. administered with 0.5 ml of the sample and then observed for 4 days. The formalized toxoid was shown to be nontoxic because mice neither died from intoxication nor showed any specific symptoms of intoxication such as muscle spasms, stiffening, or any other abnormal signs during the observation period. The toxoid was kept at 4°C until used (15, 34).

Nasal immunization and sample collection

Mice were nasally immunized at weekly intervals for 4 wk with 20 μg of BoNToxoid/A and 5 μg of mCT E112K in PBS (4, 10–13). Plasma and mucosal external secretions (nasal washes, saliva, and fecal extracts) were collected on day 28. Saliva was obtained from mice following i.p. injection with 100 μg of sterile pilocarpine (7). Fecal pellets (100 mg) were suspended into 1 ml of PBS containing 0.1% sodium azide and were then extracted by vortexing for 5 min. The samples were spun at 10,000 × *g* for 5 min, and the supernatants were collected as fecal extracts (5–7). The mice were sacrificed 7 days after the last immunization. The nasal washes were obtained by injecting 1 ml of PBS on three occasions into the posterior opening of the nasopharynx with a hypodermic needle (13).

Ab assays

Ab titers in plasma and mucosal secretions were determined by an ELISA (4–7, 11–13). Briefly, Falcon microtest assay plates (BD Biosciences) were coated with an optimal concentration of purified BoNT/A (100 μl of 2 μg/ml) in PBS overnight at 4°C (35). Two-fold serial dilutions of samples were added after blocking with 1% BSA. To detect BoNT/A-specific Ab levels, HRP-conjugated, goat anti-mouse γ or α, H chain-specific Abs were used (Southern Biotechnology Associates). For IgG Ab subclass determinations, biotinylated mAbs specific for IgG1 IgG2a, IgG2b, and IgG3 (BD Pharmingen) and peroxidase-conjugated goat anti-biotin Ab were used, as described elsewhere (11–13). Endpoint titers were expressed as the last dilution yielding an OD at 414 nm (OD_{414}) of >0.1 U above negative control values after a 15-min incubation.

Enumeration of Ab-forming cells (AFCs)

The spleens were removed aseptically and single cell suspensions were prepared, as described elsewhere (4, 10, 13). For isolation of mononuclear cells from nasal passages (NPs) and iLP, a modified dissociation method was used. This method was based upon a previously described protocol using collagenase type IV (0.5 mg/ml; Sigma-Aldrich) enzymatic dissociation to obtain single cell preparations (4, 10, 13). Mononuclear cells were purified using a discontinuous Percoll gradient (Pharmacia Fine Chemicals). Mononuclear cells at the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 (Mediatech) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS (complete medium). An ELISPOT assay was used to detect cells producing IgG and IgA Abs (5–7, 12, 13). Ninety-six-well nitrocellulose plates (Millititer HA; Millipore) were coated with 10 μg/ml BoNT/A for analysis of anti-BoNT/A-specific AFCs.

BoNT/A challenge

A 100-μl aliquot (2 μg/ml) of progenitor BoNT/A (2×10^7 i.p. LD_{50}/mg; WAKO) diluted in 0.2% gelatin/PBS was given to each mouse via the i.p. route. For oral challenge, mice were starved for 12 h before oral delivery of BoNT/A and were then gastrically intubated with a 7.5% sodium bicarbonate isotonic solution 30 min before actual intubation of BoNT/A (5–7). The various doses (25 ng, 250 ng, and 2.5 μg/mouse) of BoNT/A were gastrically intubated into the duodenum of each mice (six mice/group) using a 22-gauge ball-tip intubation needle to establish the oral LD_{50} for BoNT/A (5–7). Individual mice were monitored daily for paralysis and death.

S-IgA Ab neutralization assay

Undiluted fecal extract samples (200 μl) from mice given either BoNT/A vaccine or a nonrelevant Ag (OVA) were incubated with 50 pg of progenitor BoNT/A (WAKO) in 0.2% gelatin/PBS for 30 min at room temperature (36). The solution mixture was then injected into mice via the i.p. route, and individual mice were monitored daily for paralysis and death.

Statistics

The data are expressed as the mean ± SEM, and mouse groups were compared with control mice using a Mann-Whitney *U* test with Statview II software (Abacus Concepts) designed for Macintosh computers. A p value of <0.05 or less was considered significant.

Results

Induction of BoNT-specific S-IgA Ab responses

To date, no studies have assessed BoNT-specific mucosal IgA Ab responses. To bridge this gap in our knowledge, we first examined BoNT/A-specific mucosal immune responses in mice given nasal BoNToxoid/A plus mCT E112K as mucosal adjuvant. Nasal washes, saliva, and fecal extracts were collected 1 wk after the last nasal immunization with BoNToxoid/A plus mCT E112K and were then subjected to BoNT/A-specific ELISA. Significant S-IgA Ab responses were induced in external secretions of mice given nasal BoNToxoid/A plus mCT E112K; however, mice given nasal BoNToxoid alone did not exhibit BoNT-specific S-IgA Ab responses (Fig. 1). Interestingly, higher levels of BoNT/A-specific S-IgA Abs were detected in fecal extracts than in other mucosal secretions such as saliva and nasal washes. In contrast, nasal washes contained significant levels of anti-BoNT/A-specific IgG Abs (reciprocal $\log_2$ titer ~5). As one might expect, mice systemically immunized with BoNToxoid/A plus mCT E112K showed high titers of BoNT/A-specific plasma IgG (reciprocal $\log_2$ titer ~22), but not S-IgA Abs (data not shown). These results are the first evidence that BoNT/A-specific S-IgA Ab responses can be induced by nasal immunization with BoNToxoid plus an appropriate mucosal adjuvant (e.g., mCT E112K).

BoNT/A-specific plasma Abs in mice given the nasal vaccine

Since it has been shown that nasal immunization induces Ag-specific immune responses in systemic as well as mucosal sites, we sought to assess BoNT/A-specific plasma Ab levels after nasal vaccine delivery and found that significant responses were induced (Fig. 2*A*). The level of BoNT/A-specific IgG Abs in mice given nasal vaccine (reciprocal $\log_2$ titer ~23) was comparable to that of systemically immunized mice (reciproal $\log_2$ titer ~22). Furthermore, mice given the nasal vaccine with mCT E112K as mucosal adjuvant also showed elevated BoNT/A-specific IgG Ab levels in plasma (Fig. 2*A*), while mice given nasal BoNToxoid alone exhibited only minimally detectable levels of BoNT/A-specific plasma IgG or IgA Ab responses (Fig. 2*A*). Furthermore, mice given nasal BoNToxoid/A plus mCT E112K showed significant levels of plasma IgG1, IgG2a, and IgG2b, but not IgG3 Ab responses (Fig. 2*B*). However, anti-BoNT/A IgG2a Ab levels were lower than IgG1 and IgG2b subclass Ab responses (Fig. 2*B*). These results clearly show that our mucosal BoNT vaccine evokes

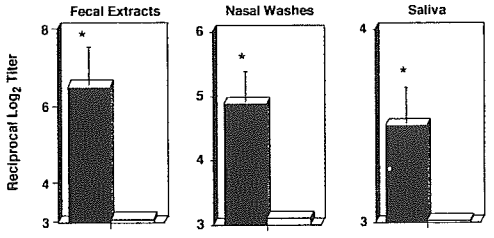

FIGURE 1. Comparison of BoNT/A-specific IgA Ab responses in nasal washes, fecal extracts, and saliva of mice given nasal BoNT vaccine with (■) or without (□) mCT E112K. Each mouse group was nasally immunized once/wk for 4 consecutive weeks with 20 μg of BoNToxoid alone or in together with 5 μg of mCT E112K as mucosal adjuvant. Seven days after the last nasal immunization, S-IgA Ab levels in nasal washes, fecal extracts, and saliva were determined by a BoNT/A-specific ELISA. The values shown are the mean ± SEM for 12 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.05$.

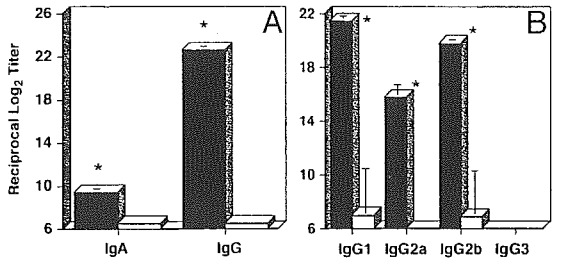

FIGURE 2. Comparison of BoNT/A-specific IgG, IgA, and IgG subclass Ab responses in plasma of mice given nasal BoNT vaccine alone (□) or together with mCT E112K as adjuvant (■). Each mouse group was nasally immunized once/wk for 4 consecutive weeks with 20 μg of BoNToxoid alone or together with 5 μg of mCT E112K as mucosal adjuvant. Seven days after the last nasal immunization, IgG or IgA (*A*) and IgG subclass (*B*) Ab levels in plasma were determined by a BoNT/A-specific ELISA. The values shown are the mean ± SEM for 12 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.01$.

two layers of BoNT/A-specific immune responses: S-IgA Abs in mucosal external secretions, and IgG and IgA Abs in plasma.

BoNT/A-specific AFCs in mice given the nasal vaccine

To confirm that BoNT/A-specific Ab responses were induced in both mucosal and systemic lymphoid tissues, mononuclear cells from NP, iLP, and spleen were taken from mice nasally immunized with BoNToxoid/A plus mCT E112K as mucosal adjuvant. These lymphoid cells were then subjected to a BoNT/A-specific ELISPOT assay to determine the numbers and isotypes of AFCs present. Mice given nasal vaccine exhibited high numbers of BoNT/A-specific AFCs in IgA effector sites such as NPs and iLP (Fig. 3). Increased numbers of BoNT/A-specific IgG AFCs were noted in NP and iLP of mice given mCT E112K as nasal adjuvant when compared with the group of mice given BoNToxoid/A alone. Furthermore, mice given BoNToxoid/A and mCT E112K as nasal adjuvant showed significant numbers of anti-BoNT IgA and IgG AFCs in spleen, while mice given BoNToxoid alone showed only minimal numbers of BoNT/A-specific IgA or IgG AFCs (Fig. 3). These results clearly show that BoNT/A-specific immunity mediated through the NALT immune system is robustly induced in both mucosal and systemic lymphoid compartments.

Induction of protective systemic immunity by the nasal BoNT vaccine

Because nasal immunization with BoNToxoid/A plus mCT E112K as mucosal adjuvant induced both mucosal and systemic Ab responses, it was important to determine whether these Ag-specific

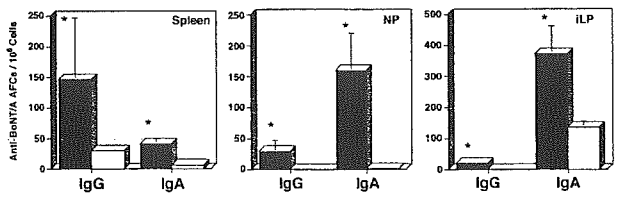

FIGURE 3. Analysis of BoNT/A-specific AFCs in mice given nasal BoNT vaccine with (■) or without (□) mCT E112K as adjuvant. Seven days after the last nasal immunization, mononuclear cells isolated from the NP, iLP, and spleen were examined using a BoNT/A-specific ELISPOT assay to determine the numbers of IgG and IgA AFCs. The results represent the mean values ± SEM for 15 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.01$.

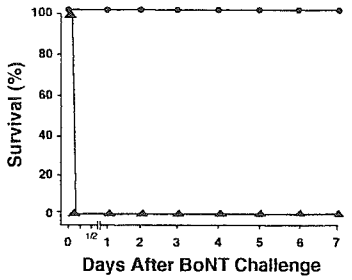

FIGURE 4. Nasal immunization with BoNToxoid/A together with mCT E112K as mucosal adjuvant elicits protection against systemic challenge with BoNT/A. Mice were immunized nasally with either 15 μg of BoNToxoid/A and 5 μg of mCT E112K (●) or 20 μg of BoNToxoid without adjuvant (▲) three times at weekly intervals. Mice were challenged with a lethal dose (4000 × i.p. LD_{50}) of BoNT/A in 0.1 ml of PBS including 0.2% gelatin via the i.p. route. Each group consisted of six mice, and the data are representative of three separate experiments.

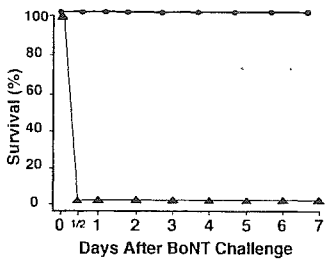

FIGURE 5. Nasal immunization with BoNToxoid/A and mCT E112K as mucosal adjuvant prevents enteric intoxication. Mice were immunized nasally with 20 μg of BoNToxoid/A and 5 μg of mCT E112K (●) or 15 μg of BoNToxoid without adjuvant (▲) three times at weekly intervals. Mice were challenged with a lethal dose (2 × oral LD_{50}) of BoNT/A in 0.25 ml of PBS including 0.2% gelatin via the oral route. Each group consisted of six mice, and the data are representative of three separate experiments.

Ab responses could protect mice from BoNT/A intoxication. To make this determination, groups of mice were immunized nasally with BoNToxoid/A plus mCT E112K or BoNToxoid without adjuvant at weekly intervals for 4 consecutive weeks. One week after the last immunization, the mice were then challenged i.p. with a lethal dose (0.2 μg/mouse: 4000 × i.p. LD_{50}) of BoNT/A, which induces paralysis and death within 30 min. Mice given nasal BoNToxoid/A plus mCT E112K as mucosal adjuvant were completely protected for the first 24 h (Fig. 4), while mice given BoNToxoid alone were not protected from paralysis and died within 24 h of challenge (Fig. 4). These findings clearly suggest that BoNT/A-specific, plasma IgG Abs in mice induced by the BoNT/A nasal vaccine were protective.

Mucosal BoNT/A-specific S-IgA Abs prevent enteric botulism

Because our nasal BoNT/A vaccine induced BoNT/A-specific S-IgA Ab responses in mucosal sites, it was important to determine whether these mucosal S-IgA Abs also provided protection against mucosal BoNT/A intoxication. To do so, we initially established an oral challenge system using gastric intubation. Because nasal washes, but not fecal extracts, contained significant levels of BoNT/A-specific IgG Abs, it was logical to devise an oral challenge system to elucidate the role of BoNT-specific S-IgA Abs for mucosal intoxication. Mice were starved and pretreated orally with 7.5% sodium bicarbonate isotonic solution before oral challenge. When various doses (25 ng, 250 ng, and 2.5 μg/mouse) of BoNT/A were administered into the duodenum of each group of mice (six mice/group), three of six mice given the highest dose (2.5 μg/mouse) died within 24 h. All mice in the other groups survived until the end of the experiment (7 days) (Table I). Thus, we established that 2.5 μg was a LD_{50} for oral challenge. Based upon these results, mice given nasal vaccine consisting of BoNToxoid/A and mCT E112K were orally challenged with a dose of 2 × LD_{50} of BoNT/A 1 wk after the last immunization. All mice in this mucosally vaccinated group survived for 7 days after oral challenge with BoNT/A (Fig. 5). Conversely, naive mice and mice given BoNToxoid alone died within 24 h after challenge (Fig. 5). These results indicate that mucosal BoNT/A-specific S-IgA Ab responses induced by our nasal vaccine containing BoNToxoid/A and mCT E112K protect mice from mucosal BoNT/A intoxication.

Mucosal BoNT/A-specific S-IgA Abs possess neutralization activity

To further establish a role for BoNT/A-specific S-IgA Abs, mice were challenged with BoNT/A pretreated with fecal extract samples. When mice were challenged with 1 × LD_{50} i.p. dose of BoNT/A that had been preincubated with a fecal extract sample, one-half survived for 3 days and one-third survived until the end of the experiment. In contrast, when mice were injected with BoNT/A and fecal extracts from mice nasally immunized with OVA plus mCT, three-quarters died of BoNT/A intoxication within 1 day (Fig. 6). These results indicate that BoNT/A-specific S-IgA Abs play a key role in the protection against BoNT/A intoxication.

Discussion

The current botulinum vaccines, both pentavalent (serotpyes A-E) and monovalent (serotype F), are used for at-risk populations such as scientists, health care providers, and the military (26, 37). Professional health care workers are well trained in avoiding mucosal BoNT intoxication while working with BoNTs. Thus, the current vaccines that induce only systemic immunity to BoNTs may be sufficient to protect these professional or military populations. However, natural botulism is disseminated by food, water, or air, and so a bioterrorist attack would most likely target mucosal surfaces. It is feared that the currently available botulinum vaccine will most likely not prevent such mucosal BoNT intoxication. Although passive immunization has been reported to prevent inhalational BoNT intoxication (38), it is not at all certain that such a systemic BoNT-specific Ab treatment would provide effective protection against food- or waterborne botulism. Furthermore, the mucosal tissue damage induced by BoNTs needs to be carefully evaluated. Our studies are aimed at developing a mucosal vaccine that

Table I. *Determination of the oral challenge dose for BoNT/A*

Oral Dose of BoNT/A (ng/mouse)	Numbers of Mice Surviving Challenge						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
25	6/6	6/6	6/6	6/6	6/6	6/6	6/6
250	6/6	6/6	6/6	6/6	6/6	6/6	6/6
2500	3/6	3/6	3/6	3/6	3/6	3/6	3/6