本試験を同時に行ってもよいので，培地性能試験は計算上，遅くとも培地調製後３週以内に行う必要がある。２週間目に行った培地性能試験結果は無菌試験中に出ることになる。ある意味では危険なやり方であるので，培地調製後できるだけ早い時期に培地性能試験を行い，本試験に臨んだ方がよいと思われる。尚，EP は培地の有効期間をバリデーションで求めることにしている。

2.5　バリデーション試験

試験は，メンブランフィルター法又は直接法の何れでも行えるが，ろ過可能な製品はメンブランフィルター法を用いることになった。バリデーション試験の一番の目的は，接種方法の選択と選択した方法で汚染菌の増殖が十分であるかどうかを指標菌を用いて検証することにある。バリデーション試験は，被検体に対する無菌試験前に，又は無菌試験と並行して行うことはできるが，可能な限り，当該品目の無菌試験前に行い，種々の問題を片付けておいた方が無難である。バリデーション試験は，1）新たな製品について無菌試験を行う場合，2）試験の実施条件に変更があった場合に行うことになる。1）については当然であるが，2）については種々のケースが考えられる。一例を挙げれば，ア）接種方法の変更，イ）不活化剤の濃度又は種類の変更，ウ）直接法における培地量の変更，エ）メンブランフィルター法におけるフィルター材質の変更，オ）培地メーカーの変更，培地調製法の変更，培地容器の変更，等々が挙げられる。抗菌剤を含む医薬品の無菌試験が一番難しい課題である。不活化ワクチンに広く用いられてきた有機水銀防腐剤（チメロサール）の不活化剤としては液状チオグリコール酸培地に含まれるチオグリコール酸（又はナトリウム塩）がその役目を担っている。そのため，国際調和は出来なかったが，水銀防腐剤添加製品の無菌試験には日局では，SCD 培地の代わりに液状チオグリコール酸培地を用い，20-25℃で培養することにした。本考え方は，米国における生物学的製剤に対する基準である CFR（Code of Federal Regulation）610.12 では規定されているが，残念ながら EP/USP には理解されなかった。日本では，これまで無菌試験法は日局，生物学的製剤基準，日本抗生物質基準で表現が異なっていたが，有機水銀防腐剤含有製剤に対する対処方法を含め，今回の改正（第十四改正第二追補）で日局に一本化できたことは，国内における無菌試験法のダブルスタンダードが解消されたことを意味する。国際調和無菌試験法を作成する段階において，抗菌剤を含む製剤をメンブランフィルター法で無菌試験を行う際の問題点についても随分議論した。最終的に，「一般に，メンブランフィルター法においては，メンブランフィルターの材質を吸着しにくいものに変更するか，洗浄液を増量するか，又は洗浄液に適当な不活化剤を加えるなど適当な方法で微生物発育阻止活性の発現を抑制する。メンブランフィルター１枚当たり，適当な界面活性剤を適量添加した洗浄液，各 100 mL で５回洗浄しても微生物発育阻止活性を抑制できない場合は，洗浄を追加することなく無菌試験を実施する」に落ち着いた。議論の中心は，洗浄液組成と洗浄回数に及んだ。洗浄

液組成に関しては，肉製又はカゼイン製ペプトンを 0.1％含む液に適当な乳化剤（例えば，ポリソルベート 80）や不活化剤（例えば，ペニシリンやセファロースポリンに対するβラクタマーゼ）を適正濃度加えてもよいとの試験者任せの表現にせざるをえなかった。洗浄回数については，当初，EP 側から洗浄液の総量は 2,000 mL を超えないような提案があった。某フィルターメーカーのデータでは 2,000 mL を超える洗浄液を用いると，膜表面にトラップされた汚染菌が洗浄液を増やすことによって膜を通過して流れ落ちる可能性があるとのことであった。EP/USP では，1 回の洗浄液量を 200 mL にしているが，日局ではフィルター 1 枚当りの洗浄液量を 100 mL にしている。これは 1 枚のフィルターを用いて被検体を処理した後，フィルターを二分してそれぞれの培地に接種することを考慮した。しかし，日局の考えは EP/USP には理解していただけなかったが，2 枚のフィルターを用いた場合には，EP/USP と同条件になるので問題はない。

2.6　製品の無菌試験

1）供試個数

ロット当りの抜取個数を表 8 に，各培地当りの最少試料採取量を表 9 に示す。何れも最少の抜取個数と採取量であるので，これらの表に示された以上の容器数や試料量を試験に用いることは構わない。注射剤については，表示量が 100 mL 以上の場合は，抜取個数を「2％又は 10 容器のうち少ない方」としている。ロット当りの製造数が少ない場合には，表 10 のようになる。尚，表示量 2.0 mL 未満の液剤は，表 8 に示した個数の 2 倍量を採取する。表 8 にある「抗生物質のバルク包装」とは，USP の注射剤通則にあるが，日本では使用されていない。複数の患者に一度に使う用量を含む包装形態のもので，清浄空気下で溶解後，一度に輸液器材等に分注して使わ

表 8　ロット当たりの抜き取り個数

ロット当たりの製造容器数	最少抜き取り個数 *
注射剤	
100 個以下	10%又は 4 容器のうち多い方
101 個以上 500 個以下	10 容器
501 個以上	2%又は 20 容器のうち少ない方
501 個以上の大容量製品	
（表示量が 100 mL 以上）	2%又は 10 容器のうち少ない方
眼剤や非注射剤	
200 個以下	5%又は 2 容器のうち多い方
201 個以上	10 容器
単回使用製品の場合は，注射剤に準じた抜き取り個数とする	
固形バルク製品	
4 容器まで	各バルク容器
5 容器以上 50 容器以下	20%又は 4 容器のうち多い方
51 容器以上	2%又は 10 容器のうち多い方
抗生物質のバルク包装（5 g 以上）[1]	6 容器
抗生物質のバルク包装（5 g 未満）	20 容器

* 1 容器あたりの内容量が両培地に接種するに十分であるなら，ここに示した容器数とする。

表 9　各培地当たりの最少試料採取量

製品の表示量	最少採取量（培地当たり）
液剤（抗生物質を除く）	
1 mL 未満	全量
1 mL 以上 40 mL 以下	半量，ただし 1 mL 以上
40 mL 超 100 mL 以下	20 mL
100 mL 超	10%，ただし 20 mL 以上
抗生物質（液剤）	1 mL
水溶性又はミリスチン酸イソプロピルで可溶性の他の医薬品	全量，ただし 200 mg 以上
懸濁または乳化して用いる非水溶性医薬品，クリームまたは軟膏剤	200 mg 以上
固形剤	
50 mg 未満	全量
50 mg 以上 300 mg 未満	半量，ただし 50 mg 以上
300 mg 以上 5 g 以下	150 mg
5 g 超	500 mg

れる。日本では使用されていないが，国際調和上，加わった事項である。

表 10　ロット当たりの製造本数が少ない場合の抜き取り個数

製造容器数	抜き取り容器数 (表示量)	
	100ml 未満	100ml 以上
～ 40	4	4
41 ～ 50	5	5
51 ～ 60	6	6
61 ～ 70	7	7
71 ～ 80	8	8
81 ～ 90	9	9
91 ～ 100	10	10
101 ～ 500	10	10
501 ～ 550	11	10
551 ～ 600	12	10
601 ～ 650	13	10
651 ～ 700	14	10
701 ～ 750	15	10
751 ～ 800	16	10
801 ～ 850	17	10
851 ～ 900	18	10
901 ～ 950	19	10
951 ～	20	10

2）培養及び観察

液状チオグリコール酸培地及び変法チオグリコール酸培地は 30 ～ 35℃で，ソイビーン・カゼイン・ダイジェスト培地は 20 ～ 25℃で 14 日間以上培養し，培養期間中に数回，及び培養最終日に菌の発育の有無を観察する。試料によって培地が混濁し，判定が困難な場合，そのほか必要な場合には，培養 14 日目に新しい培地に適量を移植し，同じ温度で元の培地とともに 4 日間以上培養する。

培地への試料の接種量は，培地量の 10%以下とする。油性医薬品を接種した培地は，観察日ごとに静かに振り混ぜる。しかし，チオグリコール酸培地を嫌気性菌の検出に使用する場合は，嫌気条件を維持するために，振り混ぜは最小限のものとする。試料によって培地が混濁し，判定が困難な場合の対応についても種々の意見が出た。日局 14 では，「試料によって培地が混濁し，判定が困難な場合，そのほか必要な場合には，新しい培地に移植し，同じ温度で 7 日間培養して観察する」となっていたが，EP/USP の主張に合わせて，「試料によって培地が混濁し，判定が困難な場合，そのほか必要な場合には，培養 14 日目に新しい培地に適量を移植し，同じ温度

で元の培地とともに4日間以上培養する」とした。ただし，EP/USPが移植量を1 mL以上としたのに対し，日局は「適量」とした。試験法の中では，具体的な観察方法については提示していないが，無菌試験において最も大事なことの一つに観察がある。人間の目で観察・判定するだけに観察には特別の注意が必要である。ごく少量の菌の発育しか認められない場合や培地表面や培地容器の底部に菌の生育が認められた場合には，細心の注意を払わなければ見逃してしまう。蛍光灯などの光を通して観察すると汚染菌の観察が容易である。また，培養途中の観察と培養最終日の観察を別人が担当することも重要である。同一者が全ての観察を行うと，どうしても観察が安易になりがちであるし，また汚染が観察された場合，無菌試験実施者と観察者が同一人の場合，正しく報告しない可能性も否定できない。

2.7 判定

以上の試験の結果，菌の発育を認めないときは，無菌試験に適合とする。菌の発育が認められたときは，不適と判定する。ただし，各種要因，汚染菌の性状などから無菌試験自体に問題があったと推測された場合には，再試験を行うことができる。再試験の結果，菌の発育が認められないときは，無菌試験に適合とする。菌の発育が認められたときは，不適と判定する。

国際調和無菌試験法では，以下の要件の一つ又は複数の事例に適合した場合には再試験を行うことができるとした。

a) 無菌試験実施環境の微生物モニタリング成績が不適の場合。

b) 試験中に用いた試験手法の評価の結果，不適の場合。

c) 陰性対照に微生物増殖が観察された場合。

d) 試験で分離された汚染菌の同定結果，汚染菌は当該無菌試験を実施するに用いた材料や技術の不適に由来するものであろうと推測された場合。

このような場合には，初回試験と同じ数量の検体を用いて再試験を行うことができるとしている。しかし，日局はEP/USPのように再試験の要件を具体的に提示することには賛成しなかった。培地調製（通常，高圧蒸気滅菌）に問題があり，対照培地に汚染菌が観察されたとする。その場合には，無菌医薬品製造のため培地調製と同じ高圧蒸気滅菌器を用いて滅菌した機器材（ゴム栓，種々の容器等）を用いて製造したロット製品は市場回収又は出荷しないことになるのか，また汚染菌の同定結果で当該汚染菌が無菌試験に由来し，製剤そのものに由来したものではないと言い切れるのか，試験中に用いた試験手法に問題があったら予めそれを報告したのかどうか，等々を考えるとEP/USPのように割り切った考え方をすることができなかった。曖昧さが残る状態で再

試験を行う場合には，分離菌の性状をそれなりに調べ，分離菌が一番増殖しやすい条件（培地，培養温度等）で，しかも検体数を増やして再試験を行うべきであると考えるが，本意見も採用されなかった。

1970年代，「無菌とは哲学的概念であり，神のみが知る」と言われてきたが，現在ではこの概念は通用しない。しかし，各出荷ロット製品のSALが10^{-3}以下であることを証明できる者はいない。要は，無菌医薬品の無菌性は製造工程で作るものであり，無菌試験で証明できるものではない。無菌試験は，飽くまでも公定試験としてセレモニー的に行っているにすぎないことを肝に銘じていただきたい。

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EBMに基づいて速効解決

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滅菌バリデーションのポイント

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ポイント 188 滅菌バリデーションの意味[1-4]を理解する.

滅菌バリデーションとは，滅菌工程が適切に行われることを科学的根拠に基づいて検証することである．滅菌バリデーションの構造を図1に示す．工業生産される医療機器の滅菌においては，国際標準化機構，第198専門委員会（ISO/TC198）で作成したヘルスケア製品の滅菌に関する国際規格と品質システム規格ISO9001/ISO13485を併せて使用するのが一般的である．

本稿では，病院における高圧蒸気滅菌を対象に滅菌バリデーションについて説明する．滅菌バリデーションは，①滅菌工程や滅菌方法を科学的な根拠に基づいて設計すること，②それらが，所期の目的どおりに再現性をもって機能することを体系的に確認すること，③文書化すること，と定義される．

ポイント 189 病院における滅菌バリデーション[1, 4-6]の流れを知る.

滅菌バリデーションは滅菌に関するすべての事項を含む．したがって，病院における滅菌バリデーションは，①滅菌物に関する製品性能確認，②滅菌器に関する事項，③滅菌終了後の滅菌物の管理に大別される．

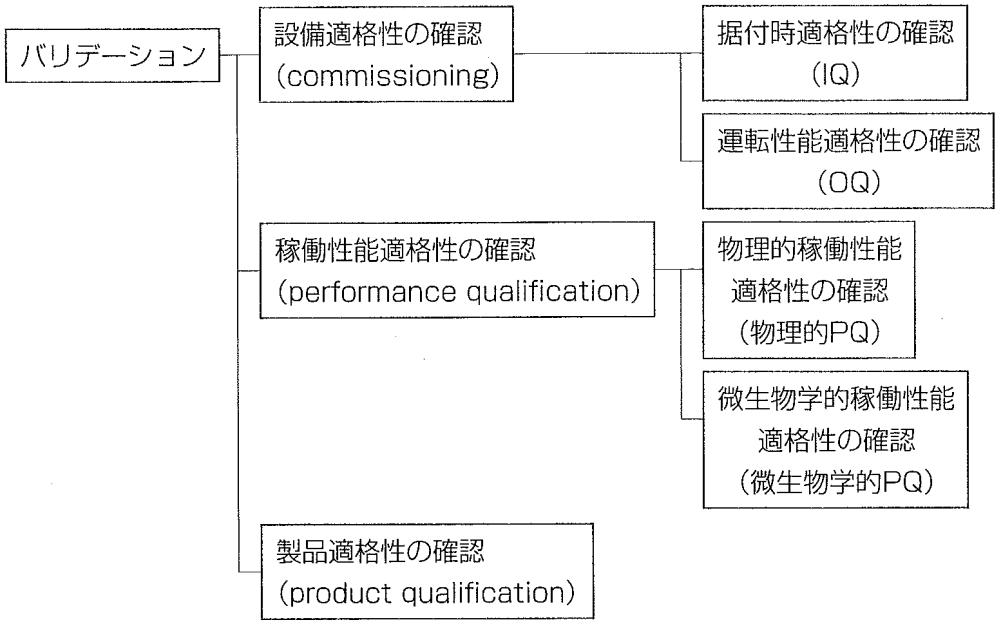

図1　滅菌バリデーションの構造[5, 6]

①製品性能確認 (product qualification)

ワーストケースにおいて，滅菌すべき製品および包装材料が滅菌条件に耐えうるものかどうかを調べる．

②滅菌器に関する事項

主に滅菌器の設置に関連する事項

据付時適格性確認（IQ：installation qualification）においては，装置が仕様どおりのものであることの確認，図面/取扱説明書等文書の確認，装置付属計器の校正，電気，水，蒸気などのユーティリティが装置の仕様に適合していること，滅菌器が正しく据え付けられていること，労働安全衛生法などの関連法規に適合することなどを確認する．

運転性能適格性確認（OQ：operational qualification）においては，無負荷運転により設置した滅菌器がメーカーの仕様どおりに作動することを確認する．そして，無負荷時において，必要な滅菌条件が所定の精度で満足されることを確認する．

稼働性能適格性確認（PQ：performance qualification）では，実滅菌物またはダミーを負荷させ，実際の運転条件において所期の性能が満たされていることを確認する．試験は，再現性確認のために少なくとも3回行う．

稼働性能適格性確認は，最大負荷と最小負荷条件下で，温度センサーを用いて滅菌物内の温度計測を行う物理的PQと，生物指標体（BI）を用いて行う微生物学的PQから成り立つ．物理的PQには化学的インジケータを使用することも含まれる．微生物学的PQは，ワーストケースにおいて目的とするSAL（無菌性保証レベル）が達成されていることを確認することである．

なお，IQ，OQ，PQにおいて滅菌器の各種計測機器類を校正（calibration）することは大切である．また，得られた情報を記録，保存すると，次に述べる滅菌バリデーションの維持管理において参照データとして役立つ．

滅菌バリデーションの維持管理

いったん確立した滅菌バリデーションの質を恒常的に維持することは，滅菌業務を適切に行うために重要である．そのために，再バリデーション，滅菌器の各種計測機器類の校正，運転記録のレビューなどを行う．

再バリデーションは，バリデーションの状況を定期的に確認する定期的再バリデーションと，滅菌工程の変更や包装材の変更などを行ったときに実施する変更時再バリデーションから成り立つ．なお，高圧蒸気滅菌バリデーションガイドラインは定期的再バリデーションを年1回以上行うことを推奨している[4]．

③滅菌終了後の管理

滅菌終了後には，滅菌物の再汚染が起こらないように注意する．さらに，滅菌日やロット番号を滅菌物に記載し，追跡性を確保することなどが重要である．

ポイント

190 滅菌器に付属した計測機器類の校正を行う[1,2]．

滅菌器には，計測機器類が付属している．たとえば，高圧蒸気滅菌器には温度計，圧力計，タイマー，これらのパラメータを記録する記録計などが設置されている．そして，これらの計測機器類を用いて滅菌工程は制御される．

そのため，もし，計測機器類が正しく作動しなければ，正確な滅菌条件を達成することができず,滅菌不良が発生する可能性がある．したがって，国家基準や国際基準に適合する標準器を用いて，計測機器類を校正しなければならない．たとえば，国家基準や国際基準に適合していることが証明されている標準温度計を用いて，滅菌器に設置された温度計を比較校正する．

なお，計測機器類が，どういう経路で校正されたかがわかり，その経路を国家基準や国際基準にまで遡（さかのぼ）れることをトレーサビリティー（traceability）という．

ポイント

191 滅菌バリデーションには，スタッフへの教育が必要である[4-6]．

滅菌バリデーションには滅菌器の保守点検，計測機器類の校正，微生物学的検査などのさまざまな内容が含まれる．そのため，滅菌器メーカーの技術員などの専門家に依頼することも多いと思われる．一方，医療現場で滅菌業務に携わるスタッフに滅菌バリデーションについて教育することも重要である．その結果，専門家の行う作業の内容や必要性などを正しく理解できるとともに，より質の高い滅菌バリデーションを実現できると考えられる．

教育内容としては滅菌器の取扱方法，保守管理，安全対策，滅菌理論，微生物学，感染管理，関連法規などが含まれる．教育方法としては，勉強会の開催や関連学会への参加などが考えられる．

さらに,資格認定の取得を奨励することは，滅菌業務に携わるスタッフの専門性や意欲を向上させる．「第一種圧力容器取扱主任者」技能講習や酸化エチレンガス滅菌に関する特定化学物質等作業主任者講習会に参加すること，第1種滅菌技師あるいは第2種滅菌技士の資格認定を取得することなどが推奨される．

知っトク！情報

ヘルスケア製品の滅菌に関する国際標準化事業[3]

近年，国際交流が盛んになるにつれ，国際標準化事業が重要になっています．

ヘルスケア製品の滅菌に関する国際標準化は，ISO/TC198において行われています．この委員会は1990年より開催されており，現在，酸化エチレンガス滅菌，放射線滅菌，蒸気滅菌，生物学的インジケータ，滅菌用語，化学的インジケータ，滅菌包装，微生物学的試験法，無菌的操作法，液状化学的薬剤滅菌，滅菌工程の一般基準，再滅菌医療用具の再生処理，洗浄消毒器に関するWG（working groups）から構成されています．

近日中に，乾熱滅菌も追加される予定です．

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Binding of *Clostridium botulinum* Type C and D Neurotoxins to Ganglioside and Phospholipid

*NOVEL INSIGHTS INTO THE RECEPTOR FOR CLOSTRIDIAL NEUROTOXINS**

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Clostridium botulinum **neurotoxins (BoNTs) act on nerve endings to block acetylcholine release. Their potency is due to their enzymatic activity and selective high affinity binding to neurons. Although there are many pieces of data available on the receptor for BoNT, little attempt has been made to characterize the receptors for BoNT/C and BoNT/D. For this purpose, we prepared the recombinant carboxyl-terminal domain of the heavy chain (H_C) and then examined its binding capability to rat brain synaptosomes treated with enzymes and heating. Synaptosomes treated with proteinase K or heating retained binding capability to both H_C/C and H_C/D, suggesting that a proteinaceous substance does not constitute the receptor component. We next performed a thin layer chromatography overlay assay of H_C with a lipid extract of synaptosomes. Under physiological or higher ionic strengths, H_C/C bound to gangliosides GD1b and GT1b. These data are in accord with results showing that neuraminidase and endoglycoceramidase treatment decreased H_C/C binding to synaptosomes. On the other hand, H_C/D interacted with phosphatidylethanolamine but not with any ganglioside. Using cerebellar granule cells obtained from GM3 synthase knock-out mice, we found that BoNT/C did not elicit a toxic effect but that BoNT/D still inhibited glutamate release to the same extent as in granule cells from wild type mice. These observations suggested that BoNT/C recognized GD1b and GT1b as functional receptors, whereas BoNT/D induced toxicity in a ganglioside-independent manner, possibly through binding to phosphatidylethanolamine. Our results provide novel insights into the receptor for clostridial neurotoxin.**

Seven types of *Clostridium botulinum* strains (A through G) are distinguished by differences in the antigenic specificity of their pharmacologically similar neurotoxins. Botulinum neurotoxins (BoNTs)[2] are synthesized as single chain peptides with a molecular mass of ~150 kDa that are proteolytically activated into a light chain (L-chain; 50 kDa) and a heavy chain (H-chain; 100 kDa) linked by a disulfide bond. It has been possible to assign some functional activities to certain domains of BoNT. The L-chain acts as a zinc-dependent endopeptidase and cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein families (1, 2), whereupon the Ca^{2+}-triggered fusion of a synaptic vesicle with the presynaptic membrane is disrupted. BoNT/B, BoNT/D, BoNT/F, and BoNT/G specifically cleave vesicle-associated membrane protein (VAMP), a membrane protein of small synaptic vesicles, at different single peptide bonds. The other BoNTs attack specific proteins of the presynaptic membrane. BoNT/A and BoNT/E cleave the 25-kDa synaptosome-associated protein (SNAP-25) at two different sites located within the carboxyl terminus (1), whereas the specific target of BoNT/C is syntaxin. BoNT/C may also cleave the carboxyl terminus of SNAP-25 (3). The H-chain is composed of two remaining domains and serves as the vehicle that delivers the L-chain into the cytosol of neuronal cells. Therefore, the extreme toxicity has to be largely ascribed to the specific binding to nerve terminals at the neuromuscular junction. The amino-terminal domain of the H-chain (H_N) is responsible for translocating the L-chain from the lumen of an acidic intracellular compartment into the cytosol subsequent to cell binding and receptor-mediated endocytosis. The carboxyl-terminal domain of the H-chain (H_C) exhibits highly selective binding for neurons, in particular for those of the cholinergic system (4). The receptor for BoNT has not been fully identified. Current evidence suggests that the receptors are composed of a certain specific ganglioside and of proteins that cooperate to form high affinity toxin-binding sites. Previously, we demonstrated the first time that BoNT/B binds specifically to synaptotagmins I and II in the presence of gangliosides GT1b and GD1a (5–7). These proteins are homologous synaptic vesicle membrane proteins thought to function as Ca^{2+} sensors for exocytosis (8). Since then, reliable data that synaptotagmin is a functional receptor protein for BoNT/B have accumulated (9). Furthermore, BoNT/G was found to interact with synaptotagmin in the absence of ganglioside (10).

In contrast to human botulism mainly involving BoNT/A, BoNT/B, and BoNT/E, BoNT/C and BoNT/D are causative agents for animal and avian botulism (11). Although *BoNT/C* and *BoNT/D* genes of several strains have been sequenced, these data appeared to be rather complicated. Some type C strains possess a unique *BoNT* gene structure that comprises two thirds of the *BoNT/C* gene and one third of the *BoNT/D* gene corresponding to the H_C portion (12, 13). Recently, we described how the gene for the mosaic form of BoNT is harbored in the isolates related to avian botulism, whereas the authentic BoNT/C may be implicated in the disease affecting mammalians, including cattle, mink, and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB200358 and AB200360.

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[2] The abbreviations used are: BoNT, botulinum neurotoxin; BSA, bovine serum albumin; HBS, HEPES-NaOH buffer; H-chain, heavy chain; H_C, carboxyl-terminal domain of the heavy chain; H_N, amino-terminal domain of the heavy chain; L-chain, light chain; PE, phosphatidylethanolamine; TLC, thin layer chromatography; VAMP, vesicle-associated membrane protein.

horses (14). Several attempts to determine the susceptibility of birds to type C and D toxins revealed that birds showed resistance to type D toxin in comparison with type C toxin (15). These findings let to us to question to what extent BoNT/C and BoNT/D share their own binding site on neural membranes, although they are immunologically related to each other (16). Direct binding experiments indicated that BoNTs adhered to a certain ganglioside such as GT1b under a low ionic strength condition, but less binding in most BoNTs was observed under a physiological condition. BoNT/C appeared to bind exceptionally to gangliosides at both low and high ionic strengths (17–20). BoNT/C caused paralysis in human neuromuscular preparation, but BoNT/D did not block the neuromuscular transmission (21). From these observations, BoNT/C and BoNT/D appear to recognize different binding sites from each other. However, no attempt has been made to isolate and characterize the binding substances for BoNT/C and BoNT/D.

In this study, we attempted to characterize the toxin binding substances for BoNT/C and BoNT/D with brain synaptosomes by treatment with various enzymes and to examine their toxic effect on cerebellar granule cells by using GM3 synthase knock-out mice. Eventually, BoNT/C and BoNT/D possess quite different characteristics in receptor recognition. Moreover, we provide informative results that BoNT/D elicits toxic action against neural cells in a ganglioside-independent manner.

EXPERIMENTAL PROCEDURES

Purification of Neurotoxins—C. botulinum type C strains CB-19, 003-9, and type D strain 1873 were used for the purification of neurotoxins (BoNT/CB-19, BoNT/003-9, and BoNT/1873, respectively). BoNT/CB-19 was reported to produce a typical type C toxin, whereas BoNT/003-9 consists of a mosaic form of type C and D neurotoxins (C/D mosaic) (Fig. 1*A*). BoNTs were purified principally according to the method of Kurazono *et al.* (22). Toxicity was assayed by the time-to-death method by intravenous injection into mice (23). Samples were also titrated by intraperitoneal injection into mice with serial 2-fold dilutions to obtain a mean 50% lethal dose (LD_{50}) by the calculations of Reed and Muench (24).

Plasmid Constructions and Recombinant Proteins—DNA fragments encoding the H_C (H_C/CB-19, amino acids 863–1291; H_C/003-9, 863–1280; and H_C/1873, 859–1276) of BoNTs were amplified with PCR using the primers 5′-CATGCCATGGCTGAATATTTCAATAATATTAATGATTCA-3′ (forward) and 5′-CCCAAGCTTTTATTCACTTACAGGTACAAAACC-3′ (reverse) for H_C/CB-19 and 5′-CATGCCATGGCTGAATATTTCAATAGTATTAATGATTCA-3′ (forward) and 5′-CCCAAGCTTTTACTCTACCCATCCTGGATC-3′ (reverse) for H_C/003-9 and H_C/1873, where the NcoI and HindIII sites were included at the ends of the forward and reverse primers, respectively. These primers were designed based on the respective DNA sequences in GenBank™ (accession numbers AB200358 for BoNT/CB-19, AB200360 for BoNT/003-9, and AB012112 for BoNT/1873). After digestion with NcoI and HindIII, the PCR products were ligated into NcoI- and HindIII-digested pET-30a vector (Novagen, Madison, WI) and verified by DNA sequencing. The recombinant plasmids were introduced into *Escherichia coli* BL21 CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). Expression of recombinant H_C was performed according to the pET system manual (Novagen). Cultures were grown at 37 °C in 100 ml of Luria-Bertani broth containing 25 μg/ml kanamycin until the optical density at 600 nm reached 0.5. After the addition of a 50 μM final concentration of isopropyl-β-D-thiogalactoside, growth was continued at 25 °C for an additional 24 h. The cells were collected and suspended in 4 ml B-PER® bacterial protein extraction reagent (Pierce) containing a protease inhibitor mixture (Sigma). After treatment with 0.1 mg/ml lysozyme for 1 h at 4 °C, the cells were solubilized on ice by sonication and centrifuged at 27,000 × *g* for 15 min at 4 °C. The supernatant was subjected to purify His_6-tagged H_C with a nickel-nitrilotriacetic Superflow column (Qiagen, Chatsworth, CA) as recommended by the instruction manual. The recombinant H_C was further purified with an anion exchange column, Mono-Q HR 5/5 (Amersham Biosciences).

Binding of Neurotoxins to Synaptosomes—The purified recombinant H_C and BoNT were radioiodinated with $Na^{125}I$ (PerkinElmer Life Sciences) by the chloramine-T method as described previously (25). The specific activities of H_Cs and BoNTs were 13–17 mCi/mg protein (26.0–34.0 MBq/nmol). After iodination, the ^{125}I-BoNTs retained >80% of that of unlabeled BoNTs. Synaptosomes were prepared from rat brain (26) and suspended in 3 mM HEPES-NaOH buffer (HBS), pH 7.0, containing 120 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. The binding of ^{125}I-H_C to synaptosomes was measured by filtration assay in the presence or absence of unlabeled H_C or BoNT (27). The concentration giving 50% inhibition (IC_{50}) was calculated using GraphPad Prism software (GraphPad Software, San Diego, CA).

Enzyme Treatments—Synaptosomes were solubilized with HBS containing 15 mM *n*-octyl-β-D-thioglucoside. Then, the solubilized synaptosomes (20 μg/ml protein) were treated at 37 °C for 30 min with 100 milliunits/ml endoglycoceramidase II (Takara Shuzo Co., Ltd., Tokyo, Japan), 100 milliunits/ml neuraminidase (Seikagaku Corp., Tokyo, Japan), and 10 μg/ml proteinase K (Wako, Osaka, Japan) or boiled for 10 min. After treatment, the remaining toxin binding activity of the solubilized synaptosomes to ^{125}I-H_C was determined by filter-absorbance assay. In brief, the solubilized synaptosomes (100 μl) were applied to absorb on MultiScreen-HA plates (Millipore Corp., Bedford, MA) for 1 h at 37 °C. After filtration, the filters were blocked with HBS and 1% BSA. After washing three times with HBS and 0.5% BSA, 0.2 ml of ^{125}I-H_C (0.5 nM) in HBS plus 0.5% BSA was added to each well and incubated at 37 °C for 1 h. Finally, the filters were washed six times with 0.25 ml of chilled HBS and 0.5% BSA. The radioactivity retained on the filter membrane was measured using a γ-counter (GMI Inc., Ramsey, MN).

TLC Overlay Assay—Lipids were extracted from lyophilized synaptosomes by the method described previously (28). Briefly, lyophilized synaptosomes were incubated with chloroform/methanol/water (20:10:1, 10:20:1, and 1:1; v/v), the volume of combined extracts being adjusted with chloroform/methanol (1:1; v/v). Extracted lipids (corresponding to 20 μg of dry tissue weight), gangliosides (GM1a, GD1a, GT1a, GD2, GD3, GD1b, GT1b, GQ1b, and GT3; 0.5 nmol each),[3] and phospholipids (phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylglycerol and phosphatidylinositol; 1 nmol each) were chromatographed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany) in chloroform/methanol/water (5:4:1) (v/v) for synaptosomal lipids and gangliosides or 65:25:4 (v/v) for phospholipids. The TLC plates were then dried and monitored for separation with resorcinol (for gangliosides) or molybdenum blue (for phospholipids). For the overlay assay, the replica plates were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) containing 1% polyvinylpyrrolidone at 4 °C for 12 h. After blocking, the plates were incubated with 1 nM ^{125}I-H_C for 2 h at room temperature. After washing three times in HBS to remove non-bound H_C, the plates were dried in air and exposed to an imaging plate (BAS-MS2340, Fuji Photo Film, Tokyo, Japan). The H_C binding lipids were detected with a FLA-3000 system (Fuji Photo Film).

[3] The ganglioside nomenclature proposed by Svennerholm was followed (see Ref. 51).

Preparation of Cerebellar Granule Neurons—Following the decapitation of 6–9-day-old C57/BL6J mice, the cerebella were removed and transferred immediately into ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline containing 5.6 mM glucose. After eliminating the meninges, the tissues were cut into 1-mm pieces and incubated in phosphate-buffered saline containing 0.25% (w/v) trypsin, 28 mM glucose, and 0.05% DNase for 20 min at 37 °C, with occasional agitation. After the removal of trypsin by brief centrifugation, the tissue was mechanically dissociated by repeated pipetting in Hanks' balanced salt solution containing 0.25% glucose, 12 mM $MgSO_4$, and 0.01% DNase. After filtering through a Falcon cell strainer (BD Labware), the cell suspension was collected by centrifugation. The pellet was resuspended in minimal essential medium containing 25 mM K^+ (HK-MEM) with 5% horse serum and 5% fetal calf serum. The cells were plated on polyethylenimine-coated four-well culture dishes (Nalge Nunc International, Naperville, IL) at a density of 4.5×10^5 cells/cm^2 and maintained at 37 °C in 5% CO_2. Between 2 and 4 days the medium was replaced with HK-MEM and 2% B-27 supplement (Invitrogen), 50 units/ml penicillin, 100 μg/ml streptomycin, and 5 μM cytosine arabinofuranoside to inhibit the replication of non-neuronal cells, and, thereafter, no further medium change was carried out before the experiment.

Exposure to BoNT and Assay of Glutamate Release—In the experiments to examine the effects of BoNT on glutamate release various concentrations of BoNT (4 μl) were added to cultures (400 μl) at 7 days and incubated at 37 °C for 18 h. BoNT-treated cerebellar cells were washed with pre-warmed low K^+ solution (10 mM HEPES-NaOH, pH 7.4, containing 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM glucose), and the extracellular solution was changed every 2 min. After changing the low K^+ solution three times, the K^+ concentration was elevated from 5 to 50 mM to depolarize the cells (29). The glutamate content of the extracellular solution was determined by reverse-phase high performance liquid chromatography on a Mightysil RP-18 GP 150-4.6 column (Kanto Kagaku, Tokyo, Japan) using precolumn derivatization with *o*-phthalaldehyde and fluorescence detection (FP-2020 Plus, Jasco, Tokyo, Japan).

Immunoblotting Detection of BoNT-cleaved Intracellular Substrates—BoNT-treated cerebellar granule cells were collected and solubilized with SDS sample buffer (0.1 M Tris-HCl, pH 6.8, containing 2% SDS, 4% glycerol, and 0.01% bromphenol blue). The samples were subjected to SDS-PAGE (12.5% acrylamide gels) and immunoblotting. The antibodies against syntaxin or VAMP-2 were obtained according to a method described elsewhere (5, 31). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Biosciences). The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 and then treated with 10 μg/ml rabbit anti-VAMP-2 antibody and mouse anti-syntaxin monoclonal antibody followed by 5 μg/ml goat anti-rabbit (or mouse) IgG conjugated with alkaline phosphatase (Bio-Rad). The reactive bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution (Promega, Madison, WI).

Others—Purified gangliosides (GM1a, GD1a, GT1a, GD2, GD3, GD1b, GT1b, GQ1b, and GT3) were kindly provided by Masao Iwamori, Faculty of Science and Technology, Kinki University. The amount of gangliosides was defined as the amount of *N*-acetylneuraminic acid determined using the method of Aminoff (30). All phospholipids were purchased from Avanti polar lipids, Inc. (Alabaster, AL). SDS-PAGE was performed in a 10 or 12.5% gel by the method of Laemmli (32). Protein concentrations were determined by the method of either Bradford (33) or Lowry *et al.* (34) with a bovine γ-globulin or BSA as a standard, respectively. C57BL/6J and ddY mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). GM3 synthase knock-out mice were generated from C57BL/6J mice according to a previously reported method (35). The neomycin resistance gene was inserted in the position of the second exon of the seven-exon *GM3* synthase gene. The mutant mice were unable to synthesize GM3 ganglioside, a simple and widely distributed glycosphingolipid. The mutant mice were viable and appeared without major abnormalities. Other detailed characteristics of the mutant mice will be reported elsewhere.

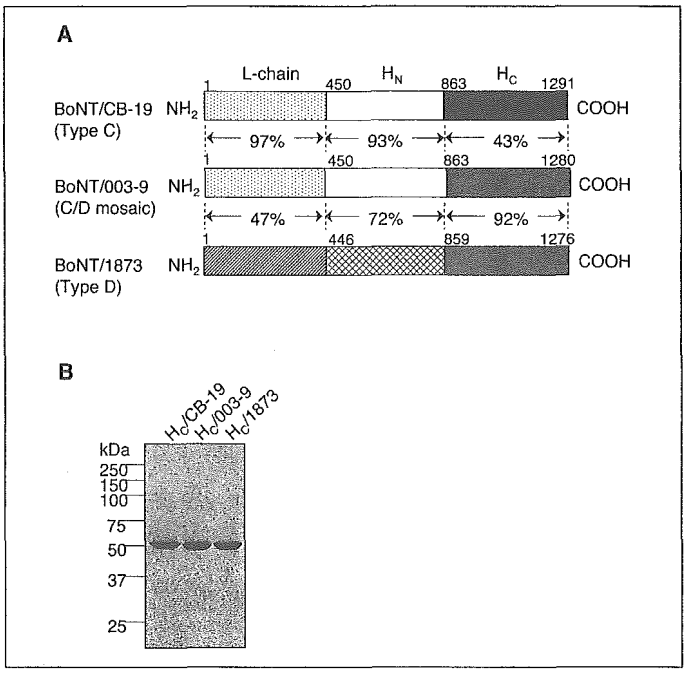

FIGURE 1. **Schematic representation of the type C and D neurotoxin.** *A*, the homology of amino acid sequences for L-chain, H_N, and H_C is shown *between* the *bars*. The *numbers above* the *bars* indicate the number of amino acid residues. The regions that show >90% homology are indicated with the same shade/pattern. C/D mosaic toxin produced by strain 003-9 consists of parts of type C and D neurotoxin. *B*, SDS-PAGE profile of recombinant H_C. Recombinant H_C (2 μg) was applied to a 10% polyacrylamide gel.

RESULTS

Binding of Recombinant H_C to Rat Brain Synaptosomes—The recombinant proteins (H_C/CB-19, H_C/003-9, and H_C/1873) were successfully obtained in a pure state (Fig. 1*B*). In the binding experiments we conducted an examination of the binding capability of ^{125}I-H_C in the presence of unlabeled homologous and heterologous H_C or BoNT (Fig. 2). The binding of ^{125}I-H_C/CB-19 was effectively inhibited in the presence of homologous H_C and the presence of BoNT/CB-19 to the same extent, whereas H_C/003-9 and H_C/1873 had little inhibitory effect on the binding. The binding of ^{125}I-H_C/003-9 and H_C/1873 were completely outcompeted by both unlabeled H_C/003-9 and H_C/1873, but not by H_C/CB-19. These data indicate that the recognition site of H_C/003-9 and H_C/1873 is the same, whereas that of H_C/CB-19 is different. When compared with the IC_{50} of the BoNTs, 003-9/BoNT appeared to possess a binding capability higher than that of BoNT/1873. BoNT/CB-19 showed a binding activity lower than did BoNT/003-9 and H_C/1873. To examine the properties of their H_C binding substances, we conducted some experiments with enzyme- and heat-treated synaptosomes for the binding of recombinant H_C (Fig. 3). In H_C/CB-19, a drastic decrease of the binding was observed in enzyme treatments with endoglycoceramidase II and neuraminidase, whereas neither proteinase K nor heat treatment affected the binding. These results may suggest that BoNT/CB-19 requires a sialic acid moiety in binding to synaptosomes, but not a proteinaceous substance. In type D binding with H_C/003-9 and H_C/1873,

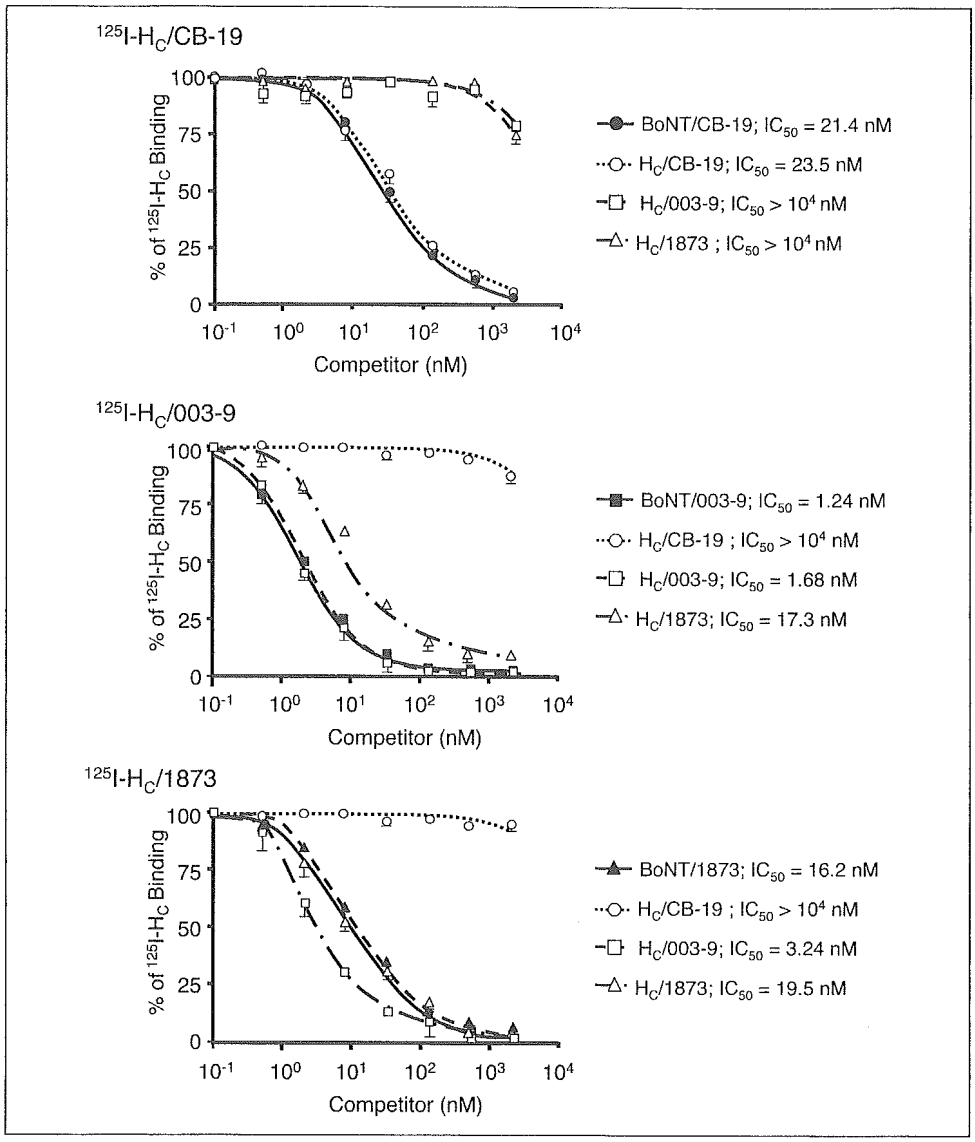

FIGURE 2. **Competition binding assay of ^{125}I-H_C/CB-19, ^{125}I-H_C/003-9, and ^{125}I-H_C/1873 to rat brain synaptosomes.** ^{125}I-H_C (0.5 nM) was incubated with synaptosomes (1 μg protein in 0.2 ml) at 37 °C for 60 min in the presence of various concentrations of unlabeled H_C and BoNT. Values are the mean ± S.D. from three experiments.

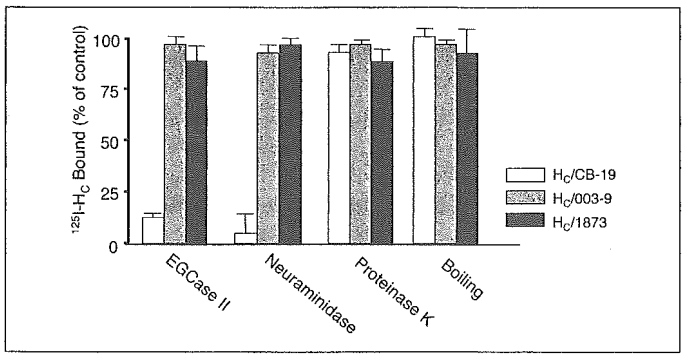

FIGURE 3. **Effect of enzyme and heat treatments on the binding of recombinant H_C to rat brain synaptosomes.** Synaptosomes were solubilized with HBS containing 15 mM *n*-octyl-β-D-thioglucoside. Then, solubilized synaptosome (20 μg protein/ml) was treated with endoglycoceramidase II (*EGCase II*) (100 milliunits/ml), neuraminidase (100 milliunits/ml), or proteinase K (10 μg/ml) for 30 min at 37 °C or boiled for 10 min. After treatment, the remaining binding activity was determined by a filter absorbance assay (see "Experimental Procedure"). The binding of H_C/CB-19 was decreased by the treatment with endoglycoceramidase II and neuraminidase, whereas the binding of H_C/003-9 and/1873 was not affected by any treatment.

there was no significant decrease in their binding after any treatment. These observations indicate that the receptors for BoNT/C and H_C/D possess characteristics different from those for type A and B, because their toxin binding to brain synaptosomes was inhibited by treatment with neuraminidase (36, 37) and boiling (data not shown). It is probable that the binding substance for BoNT/D does not involve a sialic acid moiety.

Binding of Recombinant H_C to Gangliosides and Phospholipids—In the preliminary experiments, we found that BoNT/C and BoNT/D could bind to lipids. There is little available data on the interaction of BoNT/C and BoNT/D with gangliosides and phospholipids. In the binding experiments with lipids, we performed a TLC overlay assay in the presence of 0.3 M NaCl because a nonspecific reaction was observed in the presence of 0.15 M NaCl or low concentrations. H_C/CB-19 bound to gangliosides GD1b and GT1b but not to other gangliosides or to any phospholipids. On the other hand, H_C/003-9 and H_C/1873 recognized PE, but not other phospholipids or any gangliosides (Fig. 4*A*). Analysis of brain gangliosides in the GM3 synthase knock-out mice demonstrated a pattern consistent with an absence of GM3 synthase (Fig. 4*B*, *two left sections*). The major brain gangliosides present in wild type mice (GM1a, GD1b, and GT1b) were absent in the knock-out mice. However, the GM3 synthase knock-out mice expressed major ganglioside species that comigrated with GM1b and GD1α gangliosides of the α-series, which do not require the activity of GM3 synthase (38). We then performed similar experiments using lipid extracts from mouse brain to

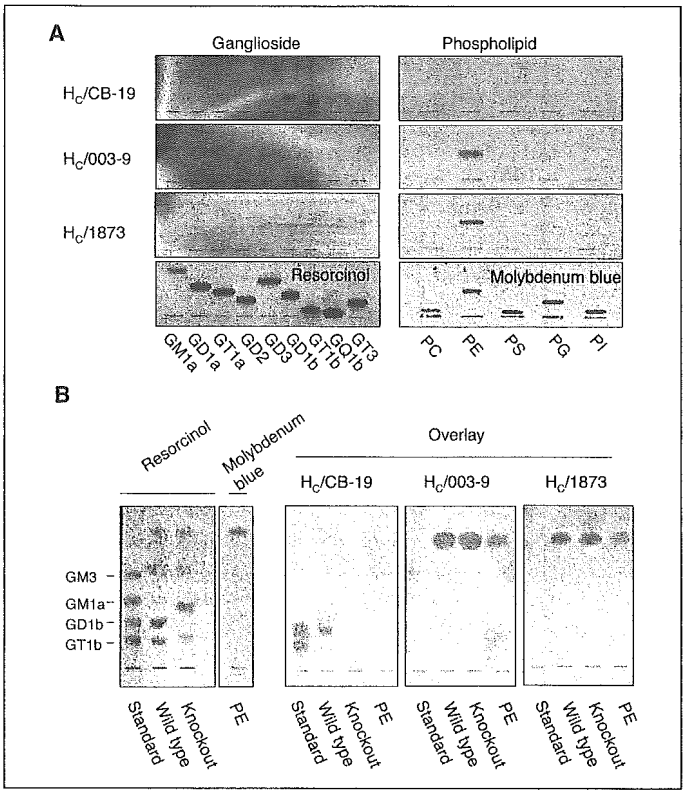

FIGURE 4. **Direct binding of ^{125}I-labeled H_C to lipids.** *A*, TLC overlay analysis to detect the direct binding of ^{125}I-labeled H_C to lipid receptors. Gangliosides (0.5 nmol) and phospholipids (1 nmol) were chromatographed on plastic-coated TLC plates. After blocking, the plates were incubated with 1 nM ^{125}I-H_C at room temperature for 2 h. The plates were then dried in air and exposed to an imaging plate. The *lowest sections* showed the detection of gangliosides and phospholipids by resorcinol and molybdenum blue reagent, respectively. H_C/CB-19 bound to GD1b and GT1b, whereas H_C/003-9 and H_C/1873 bound to PE but not to other phospholipids and any ganglioside. *PC*, phosphatidylcholine; *PS*, phosphatidylserine; *PG*, phosphatidylglycerol; *PI*, phosphatidylinositol. *B*, binding of ^{125}I-labeled H_C to the lipid extract from mouse brain. The *two left sections* show the visualization of gangliosides and PE by resorcinol and molybdenum blue reagents, respectively. The H_C-binding lipids were detected by TLC overlay assay (*three right panels*). The mixture of gangliosides GM3, GM1a, GD1b, and GT1b was used as standard.

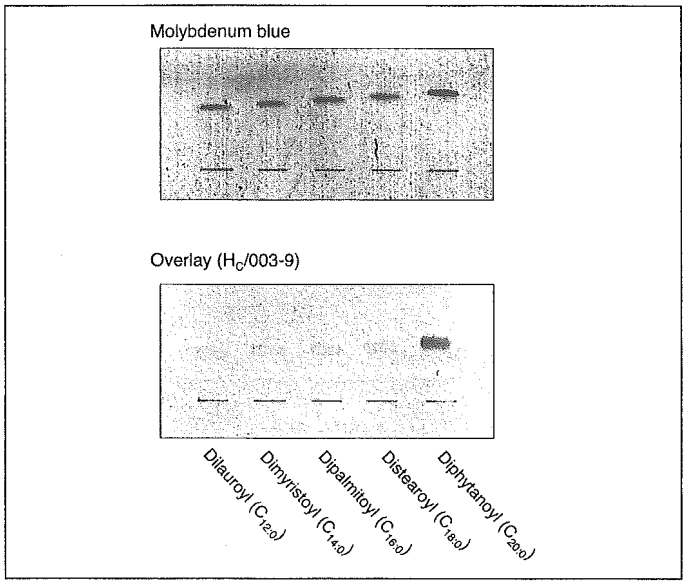

FIGURE 5. **Binding specificity of H_C/003-9 to PE.** PEs (1 nmol each) possessing different lengths of hydrocarbon tail were individually separated by TLC and overlaid as described in Fig. 4. In the *upper section* molybdenum reagent was used for the detection of PEs. The lower section shows the binding of H_C/003-9 to PEs with TLC overlay assay.

confirm the H_C binding to lipids. As shown in Fig. 4*B*, *three right sections*, H_C/CB-19 bound to two kinds of lipids showing different migration distances on TLC. The migration positions of these lipids corresponded to those of GD1b and GT1b, respectively. H_C/003-9 and H_C/1873 bound only to a lipid whose migration position in TLC was identical to that of PE. In addition, to elucidate the binding property of H_C/003-9 to PE, we determined the degree of binding to saturated fatty acid with different chain lengths of 12 to 20 carbons and found that diphytanoyl PE showed the strongest affinity with H_C/003-9 (Fig. 5).

Inhibition of Exocytosis in Mouse Cerebellar Granule Cells—To functionally evaluate the binding, we next examined the effects of BoNTs on the exocytotic release of glutamate from cerebellar granule cells prepared from GM3 synthase knock-out mice. Cerebellar granule cells release substantial amounts of glutamate in response to high K^+ treatment. In the cerebellar granule cells from wild type mice, BoNT/CB-19, BoNT/003-9 and BoNT/1873 inhibited evoked glutamate secretions in a dose-dependent manner (Fig. 6). When cerebellar granule cells from GM3 synthase knock-out mice were subjected to an examination of the effects of BoNTs, the toxic effects of BoNT/003-9 and BoNT/1873 were observed. In contrast, BoNT/CB-19 did not induce a concentration-dependent inhibition of glutamate release in the cells from knock-out mice. The content of syntaxin and VAMP-2 in cerebellar granule cells was detected by immunoblotting (Fig. 7). In the cells from wild type mice, the amount of syntaxin gradually decreased upon the addition of BoNT/CB-19 and BoNT/003-9 in a dose-dependent manner. Cleavage of VAMP-2 by BoNT/1873 was also observed. In the cells from the knock-out mouse, BoNT/003-9 affected the hydrolysis of syntaxin, and BoNT/1873 was shown to decrease the amount of VAMP-2. However, BoNT/CB-19 did not cause syntaxin to be cleaved in the cells from knock-out mice.

Sensitivity of Knock-out Mice to BoNTs—Moreover, for the purpose of evaluation of the binding to gangliosides *in vivo*, we analyzed the toxicity of BoNTs against GM3 synthase-deficient mice. The toxicity of BoNT/CB-19, BoNT/003-9, and BoNT/1873 against ddY mice were 2.1×10^7, 2.6×10^7, and 3.3×10^8 intraperitoneal LD_{50} per mg of protein. The lethalities of the three BoNTs were also determined by intraperitoneal injection into C57BL/6J mice. The specific toxicities of the three BoNTs were equivalent to those obtained in ddY mice. There was no difference in the relationship between the dose in intraperitoneal LD_{50} and the survival time with intravenous injection to death between ddY and C57BL/6J mice. When BoNT/CB-19 was injected intravenously into the knock-out mice at an appropriate dose, the survival time was prolonged and the value was decreased to 0.7% of that of the wild type mice, whereas BoNT/003-9 and H_C/1873 were still toxic to the knock-out mice and the dose (intraperitoneal LD_{50}) estimated by the survival time was 70% of that of the wild type mice (TABLE ONE).

DISCUSSION

The intracellular action of BoNT has been somewhat elucidated at the molecular level. BoNT elicits endopeptidase activity in the cell cytosol to specifically cleave proteins of the exocytotic apparatus, thereby blocking neurotransmitter release. In comparison with such intracellular events, the mechanism of receptor recognition remains obscure. In fact, many attempts have been made to identify the receptor for BoNTs, but there are only a few pieces of data reliable for BoNT/B and BoNT/G (5, 9, 10). In this study we characterized the binding of the botulinum type C and D neurotoxins. Our study had major three findings. First, both BoNT/C and BoNT/D did not require proteinaceous components for their binding to the synaptosome. Second, BoNT/C specifically bound to gangliosides GD1b and GT1b under physiological conditions, which is functionally crucial for their toxicity. Third,

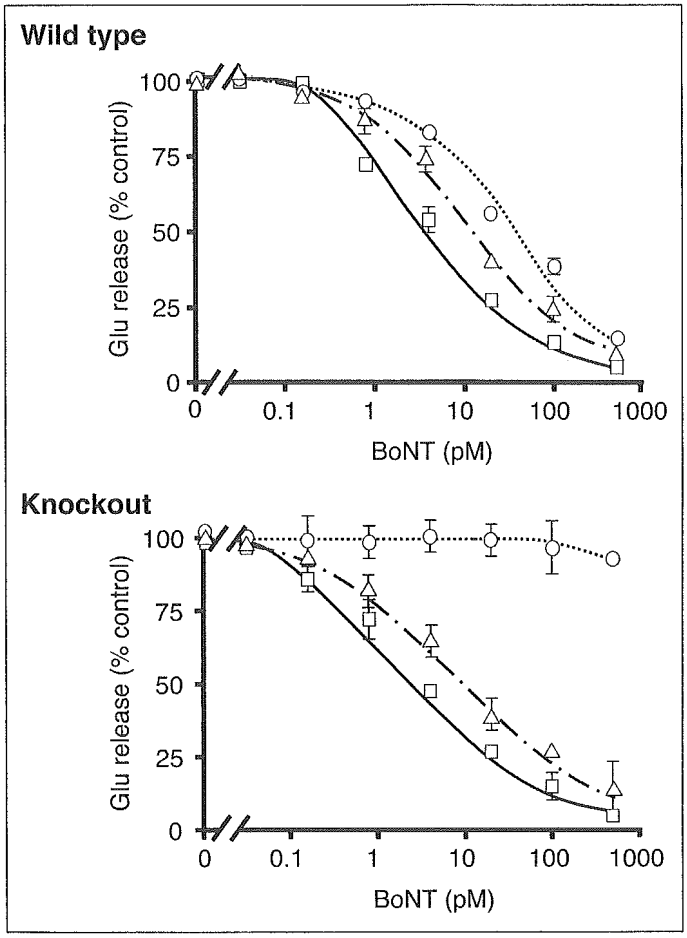

FIGURE 6. **Effects of BoNT on depolarization-evoked glutamate release from cultured cerebellar cells.** Cerebellar granule cells cultured for 7 days *in vitro*. The cells were pretreated with increasing concentrations of BoNT/CB-19 (*open circle*), BoNT/003-9 (*open square*), or BoNT/1873 (*open triangle*) at 37 °C for 18 h. After a brief washing, the cells were incubated in a low K^+ (5 mM) solution and then in the high K^+ (50 mM) solution for 2 min each. Glutamate (*Glu*) content was determined by reverse phase high performance liquid chromatography using precolumn derivatization with *o*-phthalaldehyde and fluorescence detection.

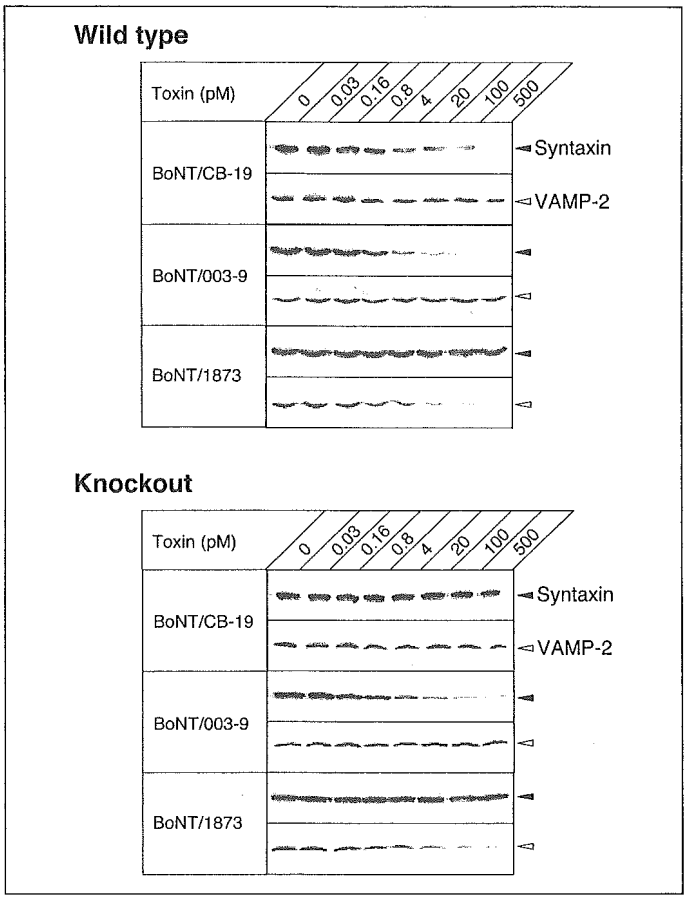

FIGURE 7. **BoNT-induced hydrolysis of syntaxin and VAMP-2 in cerebellar granule cells.** Cerebellar granule cells were exposed for 18 h in the presence of increasing concentrations of BoNT. The cells were then washed and solubilized. The samples were subjected to SDS-PAGE and immunoblotting. After blocking the nitrocellulose membrane, it was treated with 10 μg/ml rabbit anti-VAMP-2 antibody and mouse anti-syntaxin antibody followed by 5 μg/ml goat anti-rabbit (or mouse) IgG conjugated with alkaline phosphatase. The reactive bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution.

BoNT/D specifically bound to PE under physiological conditions, which was in a hydrocarbon tail-dependent manner. We surveyed the binding capability of BoNT/C and BoNT/D to the solubilized fraction from rat brain synaptosomes. Protease treatment and boiling were ineffective in decreasing the toxin binding capability for BoNT/C and BoNT/D, indicating that their receptors were not involved in the proteinaceous property. In fact, the TLC overlay assay revealed that H_C/C (CB-19) effectively bound to gangliosides GD1b and GT1b and that H_C/D (003-9 and 1873) bound to PE in the presence of 0.3 M NaCl. It has been reported that BoNT/A and BoNT/B bound directly to a certain gangliosides, but the binding was strongly affected in the presence of >0.05 M NaCl. In the direct binding with TLC, BoNT/A was not found to interact with ganglioside GT1b under a physiological ionic strength (18).

To clarify ganglioside dependence in the binding of BoNT/C and BoNT/D, we examined their toxic effects with cerebellar granule cells from GM3 synthase knock-out mice. In the mice, complex gangliosides including GD1b and GT1b were absent in the extract of brain synaptosomes. As expected, treatment of BoNT/C on the granule cells from the knock-out mice did not cause the reduction of the K^+-evoked glutamate release. On the other hand, C/D mosaic toxin and BoNT/D still elicited an inhibitory effect on glutamate release in the granule cells from knock-out mice to the same extent as in those from wild type mice. These results suggested that gangliosides require an essential receptor component for BoNT/C, as well as the other type of BoNTs, whereas type D toxin induces toxicity to target cells in a ganglioside-independent manner. The ganglioside dependence on the binding of BoNT/C to neural cells seems to be higher than the other type of BoNTs, because binding of the other BoNT to gangliosides was affected in the presence of NaCl (17–19). In the *in vivo* experiments, we confirmed that the knock-out mice were more resistant to BoNT/C than wild type mice because the knock-out mice did eventually succumb to the toxin, but they were still sensitive to BoNT/D. These observations, together with the results of *in vitro* binding experiments, indicated that GD1b and GT1b were major functional components of the receptor for BoNT/C. It is possible, although perhaps unlikely, that another ganglioside might be the functional receptor. These results also support a notion that BoNT/C and BoNT/D possess quite different properties in regard to ganglioside dependence.

The H_C fragment derived from the C/D mosaic and BoNT/D adhered specifically to PE. Although the physiological significance of PE as the binding component for H_C/D remains to be elucidated, there are several reports that PE has been described as a putative receptor for pathogenic bacteria, including *Chlamydia pneumoniae* and *Chlamydia trachomatis* (39), *Helicobacter pylori* (40), *Helicobacter mustelae* (41), *Haemophilus influenzae* (42), *Campylobacter upsaliensis* (42), enteropathogenic and enterohemorrhagic *E. coli* (43), and *Actinobacillus pleuropneu-*

TABLE ONE

Sensitivity of wild type and knock-out mice to BoNT/CB-19, BoNT/003-9, and BoNT/1873

BoNT	Mouse	Survival time[a]	Toxicity[b]	
			$\times 10^2$ ip[c] LD_{50}/ml	Percentage
		min		%
CB-19	Wild type	53 ± 2	1,040	100
	Knock-out	312 ± 13	7	0.7
003-9	Wild type	49 ± 3	1,290	100
	Knock-out	57 ± 5	889	69
1873	Wild type	48 ± 1	3,330	100
	Knock-out	54 ± 4	2,410	72

[a] Data are presented as the mean ± S.D. (n = 3).
[b] The toxicity was determined by the time-to-death method by intravenous injection of 0.1 ml of BoNT/CB-19 (5 μg/ml), BoNT/003-9 (5 μg/ml), and BoNT/1873 (1 μg/ml).
[c] Intraperitoneal.

moniae (44). The binding of these bacteria to PE is thought to mediate attachment to host cells and subsequent infection. Because PE is a widely distributed phospholipid in the plasma membrane, being primarily on the cytosolic leaflet of the bilayer (45), it might be difficult to function as a neurospecific receptor. From these observations, two points are worthy of consideration. First, PE is predominantly a component of the inner leaflet of the plasma membrane bilayer (46). However, recent studies have revealed that PE moved to outward of membrane mediated by ATP-binding cassette transporters (47). Second, a wide disparity in the binding ability of *H. pylori* to PE from different sources was observed (40), suggesting that the hydrocarbon tail in PE plays a role in the possible receptor function of PE. In fact, H_C/003-9 strongly bound to PE consisting of a longer hydrocarbon tail ($C_{20:0}$), indicating that hydrophobicity is important for BoNT/D binding. Moreover, it was reported that PE composed of a long hydrocarbon tail mainly existed in neurons (48, 49). Ganglioside-independent binding of BoNT/D seems to accord with the results reported by Knight *et al.* (50). They found that the type D toxin, but not the type C toxin, blocked catecholamine release in a bovine adrenal medullary cell culture.

In this study we provide a new, informative idea on the receptors of BoNT/C and BoNT/D. Our present data cannot exclude the possibility that the representative substances to be defined as type C- or type D-specific binding are still present, but such substances must be extremely resistant to protease and heat treatment. The properties of the receptor for BoNT/D as well as that for the C/D mosaic seem to be quite different from those of the other BoNTs. This indicates a critical issue to be resolved in regard to the question as to why BoNT/D and the C/D mosaic elicit a toxic effect on animals and birds specifically, but not on humans.

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