Characterization of the Receptor-binding Domain of Tetanus Toxin*

(Received for publication, August 3, 1992, and in revised form, January 14, 1993)

Jane L. Halpern† and Anne Loftus
From the Division of Bacterial Products, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

The carboxyl-terminal half of the heavy chain of tetanus toxin (Hc) contains the domain required for binding to purified gangliosides and neuronal cells. The structural requirements for the interaction of Hc with receptor were studied by generating mutants of Hc with deletions at either the carboxyl or amino terminus and characterizing their binding. A deletion of 10 or more amino acids from the carboxyl terminus resulted in a major loss of Hc binding to purified gangliosides and spinal cord neuronal cells, whereas a deletion of the carboxyl-terminal 5 amino acids did not affect binding. The removal of up to 263 amino acids from the amino terminus did not inhibit binding. Each of the truncated proteins was much more sensitive to trypsin than was full-length Hc, suggesting an alteration in conformation. The receptor binding activity of Hc was not retained in a peptide corresponding to the carboxyl-terminal 20 amino acids. These data suggest that the carboxyl-terminal region of Hc is important for maintaining a conformation necessary for binding to receptor.

Tetanus toxin is a protein neurotoxin of 1315 amino acids produced by Clostridium tetani (1, 2). Intoxication of neuronal cells by tetanus toxin results in the inhibition of neurotransmission; however, the mechanism of this process remains unclear (for recent reviews, see Refs. 3 and 4). Inhibition of neurotransmission by tetanus toxin is believed to require at least three steps: 1) binding to a eukaryotic cell receptor, 2) translocation to the cytosol, and 3) disruption of the secretory pathway.

Tetanus toxin is synthesized as a single polypeptide chain that is cleaved by clostridial proteases (5) to yield two fragments, the light chain derived from the amino terminus and the heavy chain derived from the carboxyl terminus (6). The two chains are linked by a single disulfide bond and by strong noncovalent interactions. Treatment of the heavy chain with papain or other proteases results in proteolysis at one additional site (7), generating two fragments, the light chain linked by a disulfide bond to approximately one-half of the heavy chain and the carboxyl-terminal half of the heavy chain (Hb). Each of the three fragments of tetanus toxin appears to represent a functional domain necessary for different steps in intoxication. The light chain is responsible for intoxicating target cells (8–10), and the amino-terminal half of the heavy chain has been proposed to translocate tetanus toxin across membranes (11, 12). Hc is required for recognition of and binding to target cells (13). This fragment also retains the ability of intact tetanus toxin to undergo retrograde axonal transport (13, 14).

The precise structure of the receptor for tetanus toxin has not been identified. Tetanus toxin binds to certain gangliosides present on neuronal cells, and many studies indicate that these gangliosides may act as the cellular receptor for tetanus toxin (3, 4, 15–18). It has been proposed that tetanus toxin must also interact with an additional receptor in order for intoxication to proceed (19). A more complete characterization of the structure of Hc required for binding may be useful for understanding initial steps in intoxication. In this study, we have constructed mutants of Hc with defined deletions and characterized the binding of these proteins to purified gangliosides and neuronal cells.

EXPERIMENTAL PROCEDURES

Reagents—[35S]Metionine (>1000 Ci/mmol) was purchased from Du Pont-New England Nuclear. Oligonucleotides were purchased from Lofstrand Laboratories (Gaithersburg, MD) or synthesized on a Milligen Cyclone 2 oligonucleotide synthesizer (Millipore Corp., Bedford, MA). GTβ was purchased from Matreya Inc. (Pleasant Gap, PA). Hc was expressed in Escherichia coli cells and purified as described (20). Hc was labeled with [35S]-labeled Bolton-Hunter reagent (Du Pont-New England Nuclear) to a specific activity of ~2 Ci/μg (20). Trypsin was from Fromega.

Synthesis and in Vitro Expression of Hc Genes—DNA fragments coding for defined regions of Hc were amplified by the polymerase chain reaction using plasmid SS1261 as template. This plasmid is a derivative of pTTQ8 and contains the coding sequence for amino acids 888–1315 of tetanus toxin (20). For each amplification reaction, one primer consisted of 25 base pairs homologous to the desired 5' end of the open reading frame plus a stop codon, a PstI site, and 3 additional base pairs. As an example, the primers for amplification of full-length Hc had the sequences 5'-GCGCGAATACGACTCACTAT- and 3'-GCGCTGCAGTCATGAACATATCAATCTGTTTAATC-3'.

The polymerase chain reaction products were transcribed in vitro using an mCAP RNA capping kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). After transcription, the mRNA was purified using an RNAid kit (Bio 101, La Jolla, CA) and stored in RNase-free water. The mRNA was translated in a rabbit reticulocyte lysate system (Fromega) according to the manufacturer's instructions. The final reaction mixture (100 μl) contained 1 μM [35S] methionine (8 μCi). An aliquot from each translation reaction was diluted into SDS sample buffer containing 50 mM dithiothreitol and 1% SDS, heated to 90 °C for 3 min, and analyzed by SDS-polyacrylamide gel electrophoresis (21) on 10–20% gradient polyacrylamide gels obtained from Integrated Separation Systems (Hyde Park, MA).
 Autoradiography was performed by exposing the dried gels to XAR-5 film (Kodak). The concentration of each product formed was determined on a separate aliquot by measuring the incorporation of $^{35}$S-methionine into trichloroacetic acid-precipitable material.

**Preparation of Fetal Mouse Spinal Cord Dorsal Root Ganglion Neurons**—Cultures of fetal mouse spinal cord dorsal root ganglion neurons were prepared by a previously published procedure and grown in 35-mm diameter dishes for 2–3 weeks (22).

**Buffered saline (PBS)**—1.0 mM KH$_2$PO$_4$, 5.0 mM Na$_2$HPO$_4$, pH 7.5, 0.15 M NaCl, and diluted as indicated in recording medium (10 mM Hapes, 4.135 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM CaCl$_2$, 1 mM MgCl$_2$, containing 1.0 mg/ml ovalbumin. Spinal cord cultures were rinsed twice with recording medium and incubated for 90 min at 4°C with the translation products. The dishes were washed six times with recording medium (2 ml/wash), solubilized in 0.1 M NaOH, and counted in a liquid scintillation counter. Each experiment was done in duplicate and repeated at least two times. A previously confirmed (23), the inclusion of a 200-fold excess of unlabeled tetanus toxin or Hc during the incubation did not inhibit binding of $^{35}$S-labeled translation products.

**Ganglioside Binding Assay**—Microtiter plates were coated with GTlb by adding 0.1 ml of methanol containing 0.1 μg of ganglioside to each well and allowing the wells to dry. The plates were incubated with PBS containing 0.5% ovalbumin (binding buffer) for 1 h to block remaining protein-binding sites. The translation products were diluted into binding buffer, added to individual wells, and incubated for 2 h at room temperature. The plates were washed six times with PBS, and individual wells were counted in a liquid scintillation counter. The amount of material that bound to wells in the absence of ganglioside was defined as nonspecific binding and has been subtracted. Nonspecific binding ranged from 5 to 20% of total binding. The nonspecific binding as measured by determining the amount of $^{35}$S-labeled Hc proteins bound to ganglioside-coated microtiter plates in the presence of an excess amount of unlabeled Hc or tetanus toxin was ~20% of total binding. Certain components of the rabbit reticuloocyte lysate from the translation reaction appeared to inhibit the binding of tetanus toxin-derived proteins in this assay. At the concentration of lysate present in the binding assay (10–20%), binding was inhibited by 10–30% by different lots of lysate. Each experiment was done in duplicate and repeated at least three times.

**Enzyme-linked Immunosorbent Assay**—Microtiter plates were coated with GTlb, by adding 0.1 ml of methanol containing 0.1 μg of ganglioside to each well and allowing the wells to dry. The plates were incubated with PBS containing 0.5% ovalbumin (binding buffer) for 1 h to block remaining protein-binding sites. The translation products were diluted into binding buffer, added to individual wells, and incubated for 2 h at room temperature. The plates were washed six times with PBS, and individual wells were counted in a liquid scintillation counter. The amount of material that bound to wells in the absence of ganglioside was defined as nonspecific binding and has been subtracted. Nonspecific binding ranged from 5 to 20% of total binding. The nonspecific binding as measured by determining the amount of $^{35}$S-labeled Hc proteins bound to ganglioside-coated microtiter plates in the presence of an excess amount of unlabeled Hc or tetanus toxin was ~20% of total binding. Certain components of the rabbit reticuloocyte lysate from the translation reaction appeared to inhibit the binding of tetanus toxin-derived proteins in this assay. At the concentration of lysate present in the binding assay (10–20%), binding was inhibited by 10–30% by different lots of lysate. Each experiment was done in duplicate and repeated at least three times.

**Preparation of Antibodies**—A peptide with the sequence CDKILGCDWYFVPTDEGWTND was synthesized using an Applied Biosystems Model 430A peptide synthesizer according to the manufacturer's instructions and purified by high performance liquid chromatography. An amino acid analysis was performed to confirm the composition of the peptide. This peptide corresponds to the carboxy-terminal 20 amino acids of tetanus toxin (residues 439-458 of Hc) plus a cysteine incorporated at the amino terminus for conjugation to a carrier protein. The conjugate was prepared by dissolving 10 mg of bovine serum albumin in 1.0 ml of 0.1 M NaHCO$_3$, pH 8.0, and transferring this mixture to a vial containing 5 mg of m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co.). This mixture was incubated at room temperature for 15 min; 10 mg of peptide were added; and the incubation was continued, with stirring, for an additional 3 h. The conjugate was dialyzed against PBS and stored at −20°C. A rabbit was immunized subcutaneously with ~50 μg of peptide-bovine serum albumin conjugate in Freund's complete adjuvant and boosted 3 and 6 weeks later with the same quantity in Freund's incomplete adjuvant. The rabbit was bled 3 weeks after the second boost, and antibodies specific for the immunizing peptide were purified by passing the serum over a tetanus toxoid affinity column (20). A polyclonal antibody against Hc was produced and purified using similar methods.

The production and characterization of monoclonal antibodies 18.1.7 and 18.2.12.6 have been described (24). These antibodies were raised against tetanus toxoid and recognize epitopes on Hc. Monoclonal antibody 18.1.7 inhibits and monoclonal antibody 18.2.12.6 enhances the binding of tetanus toxin and Hc to gangliosides and neuronal cells (24).

**RESULTS**

DNA templates coding for different regions of Hc were prepared to identify specific regions involved in receptor binding. Incorporation of a T7 RNA polymerase promoter and a start codon at the 5' end of these templates allowed them to be transcribed and translated in vitro. The structure of each of the proteins produced by this method is outlined schematically in Fig. 1. Hc produced by this technique comprises the carboxy-terminal 458 amino acids of tetanus toxin that correspond to Hc derived from tetanus toxin by proteolysis with papain plus 8 additional amino acids from the amino-terminal half of the heavy chain. Hc contains one internal disulfide bond between cysteines 220 and 236. Each of the genes was translated in a reaction using $^{35}$S-methionine and analyzed by SDS-PAGE and autoradiography (Fig. 2). The main product from each translation reaction migrated as a band of the predicted molecular weight. Some of the translation reactions contained additional low molecular weight bands, which may be proteolytic breakdown products or result from premature termination. The mobility of Hc was identical to that of intact Hc. The disulfide bond in Hc between cysteines 220 and 236 is present in each of the proteins except Hc$_{263-458}$. Hc contains 6 methionine residues at positions 92, 89, 123, 196, 292, and 385.
to that of purified recombinant Hc. Each of the genes was transcribed and translated with approximately equal efficiency; yields ranged typically from 0.02 to 0.1 μg/ml.

The translation product coded for by the full-length synthetic Hc gene appeared to retain the biological characteristics of tetanus toxin-derived Hc (Table I). Hc bound to GTlb immobilized on microtiter plates. This binding was inhibited by monoclonal antibody 18.1.7 and enhanced by monoclonal antibody 18.2.12.6, indicating that the epitopes recognized by these antibodies were intact. Hc was also found to undergo retrograde axonal transport similarly to toxin-derived Hc (data not shown). Each of these activities could be measured without purification of Hc from the lysate reaction mixture.

Hc proteins truncated at either the carboxyl or amino terminus of Hc were examined for ganglioside binding activity (Fig. 3). Each of the proteins with an amino-terminal deletion bound to ganglioside-coated plates. A deletion of 5 amino acids from the carboxyl terminus (HC1-445) had no effect on ganglioside binding; however, deletions of 10 (HC1-446) or 23 (HC1-469) carboxyl-terminal amino acids completely abolished ganglioside binding.

Since the region of Hc required for binding to cellular receptors may differ from that required for binding to purified gangliosides, each of the truncated Hc proteins was examined for binding to cultured neuronal cells (Fig. 4). Binding to the neuronal cells paralleled the ganglioside binding. Each of the

| Peptide | Hc-ganglioside complex | 20000 | 30000 |
|---------|------------------------|-------|-------|
| None    | 874 ± 92               |       |       |
| mAb 18.2.12.6 | 2591 ± 5       |       |       |
| mAb 18.1.7   | 142 ± 17             |       |       |

* Monoclonal antibody.

**Effect of anti-Hc monoclonal antibodies on [35S]-labeled Hc binding to gangliosides**

The translation product coded for by the full-length synthetic Hc gene appeared to retain the biological characteristics of tetanus toxin-derived Hc (Table I). Hc bound to GTlb immobilized on microtiter plates. This binding was inhibited by monoclonal antibody 18.1.7 and enhanced by monoclonal antibody 18.2.12.6, indicating that the epitopes recognized by these antibodies were intact. Hc was also found to undergo retrograde axonal transport similarly to toxin-derived Hc (data not shown). Each of these activities could be measured without purification of Hc from the lysate reaction mixture.

Hc proteins truncated at either the carboxyl or amino terminus of Hc were examined for ganglioside binding activity (Fig. 3). Each of the proteins with an amino-terminal deletion bound to ganglioside-coated plates. A deletion of 5 amino acids from the carboxyl terminus (HC1-445) had no effect on ganglioside binding; however, deletions of 10 (HC1-446) or 23 (HC1-469) carboxyl-terminal amino acids completely abolished ganglioside binding.

Since the region of Hc required for binding to cellular receptors may differ from that required for binding to purified gangliosides, each of the truncated Hc proteins was examined for binding to cultured neuronal cells (Fig. 4). Binding to the neuronal cells paralleled the ganglioside binding. Each of the

**TABLE I**

**Effect of anti-Hc monoclonal antibodies on [35S]-labeled Hc binding to gangliosides**

The translation product coded for by the full-length synthetic Hc gene appeared to retain the biological characteristics of tetanus toxin-derived Hc (Table I). Hc bound to GTlb immobilized on microtiter plates. This binding was inhibited by monoclonal antibody 18.1.7 and enhanced by monoclonal antibody 18.2.12.6, indicating that the epitopes recognized by these antibodies were intact. Hc was also found to undergo retrograde axonal transport similarly to toxin-derived Hc (data not shown). Each of these activities could be measured without purification of Hc from the lysate reaction mixture.

Hc proteins truncated at either the carboxyl or amino terminus of Hc were examined for ganglioside binding activity (Fig. 3). Each of the proteins with an amino-terminal deletion bound to ganglioside-coated plates. A deletion of 5 amino acids from the carboxyl terminus (HC1-445) had no effect on ganglioside binding; however, deletions of 10 (HC1-446) or 23 (HC1-469) carboxyl-terminal amino acids completely abolished ganglioside binding.

Since the region of Hc required for binding to cellular receptors may differ from that required for binding to purified gangliosides, each of the truncated Hc proteins was examined for binding to cultured neuronal cells (Fig. 4). Binding to the neuronal cells paralleled the ganglioside binding. Each of the

**DISCUSSION**

Tetanus toxin binds to neuronal cells through the Hc region derived from the heavy chain. We have analyzed the binding properties of Hc truncated at both the amino and carboxyl...
FIG. 5. Trypsin treatment of Hc proteins. Each translation product (1 μl, ~10,000 cpm) was incubated with (+) or without (−) trypsin (0.2 μg/ml final concentration) in a total volume of 200 μl of PBS at 37 °C for 5 min. The reactions were stopped by the addition of trichloroacetic acid. Samples were centrifuged at 3000 × g for 30 min, resuspended in SDS sample buffer, and analyzed by SDS-PAGE.

FIG. 6. Binding of anti-peptide 439-458 and anti-Hc to tetanus toxin. The binding of anti-peptide 439-458 (hatched bars) and anti-Hc (shaded bars) to tetanus toxin bound to G₀-coated microtiter plates was measured as described under "Experimental Procedures."

termini to characterize the binding domain more precisely. The removal of 10 amino acids from the carboxyl terminus of Hc resulted in a loss of binding to both purified gangliosides and neuronal cells, whereas removal of up to 263 amino acids from the amino terminus did not affect binding, suggesting that the carboxyl terminus is critical for binding. This loss of binding associated with truncations at the carboxyl terminus may be interpreted in different ways. This region may mediate binding by direct interaction with a ganglioside receptor. Another possibility is that the carboxyl-terminal amino acids are required to maintain Hc in a conformation necessary for binding.

Several lines of evidence suggest that the carboxyl-terminal amino acids do not constitute the entire ganglioside-binding domain. In a simple model in which Hc binds to gangliosides through the extreme carboxyl terminus, the removal of part of this region could result in some alteration of ganglioside binding activity. The removal of the final 5 amino acids from Hc had no effect on binding, indicating that if the carboxyl terminus of Hc contains a ganglioside-binding domain, it does not include the final 5 amino acids.

Studies done using a synthetic peptide and an antibody against this peptide also support the hypothesis that the carboxyl-terminal amino acids do not, without additional regions of Hc, mediate binding to gangliosides. Peptide 439-458 did not bind to gangliosides or inhibit the binding of Hc even at millimolar concentrations. This suggests that if this
peptide binds to ganglioside, its affinity is too low to be measured in this assay.

Polyclonal antibodies against peptide 439–458 were able to bind to Hc that was already bound to ganglioside, indicating that these antibodies react with at least one site that is distinct from the ganglioside-binding site. Anti-peptide 439–458 did not appear to compete with Hc for binding to ganglioside, suggesting that this region of Hc may not interact directly with ganglioside. Since anti-peptide 439–458 is a polyclonal antibody, this assay may not detect the presence of a small population of antibodies that bind to Hc competitively with ganglioside. We are preparing monoclonal antibodies to this peptide to investigate this possibility.

Each of the truncated proteins displayed greatly increased susceptibility to proteolysis with trypsin, most likely due to changes in conformation. This experiment was not able to detect conformational differences between the nonbinding and ganglioside-binding mutants, suggesting that the entire primary sequence is important in order for Hc to correctly fold into a protease-resistant conformation. Our data indicate that removal of different amino acids from Hc may result in a number of different conformations, of which only some remain ganglioside binding activity. We are presently investigating if techniques other than proteolytic digestion may be able to distinguish these conformations.

Hc contains one disulfide bond between cysteines 220 and 236 (25). The presence of this disulfide bond appeared not to be important for binding since Hc220-436, which lacks both cysteine residues, retained ganglioside binding activity. Whereas amino acids 1–963 are not required for binding, this region is clearly important for proper folding of Hc, as indicated by the proteolysis experiments. This region may also be involved in events that occur subsequent to receptor binding, such as internalization or retrograde axonal transport. It is not known if binding to ganglioside is sufficient to trigger the retrograde axonal transport of Hc or if internalization into vesicles and transport is a more complex process. The truncated proteins reported in this study may be useful for studying the requirements for retrograde axonal transport.

The involvement of the carboxyl-terminal region in receptor binding has been reported previously for several bacterial toxins. The carboxyl-terminal amino acids of protective antigen, the receptor-binding subunit of anthrax toxin, were found to be essential for binding to macrophages (26). A peptide containing the carboxyl-terminal 54 amino acids of diphtheria toxin, generated by hydroxylamine, was demonstrated to block binding of the toxin to Vero cells and to protect against its lethal effect (27). The receptor binding activity of the enterotoxin from Clostridium perfringens was localized to the carboxyl-terminal 31 amino acids (28). In contrast to our results with tetanus toxin, the binding activities of both diphtheria toxin and enterotoxin were retained on a relatively small peptide. The ability of Hc-derived proteins to bind to purified gangliosides paralleled their binding to spinal cord neuronal cells. Because the binding of these proteins to neuronal cells was not inhibited by excess unlabelled tetanus toxin, it is not possible to determine if the association of Hc1-448 and Hc1-455 with the cells represents specific or nonspecific binding. This inability to demonstrate specific saturable binding of tetanus toxin under physiological conditions has been reported previously (23) and has been attributed to the presence of a large number of low affinity binding sites present on the plasma membrane. The greatly decreased binding to neuronal cells of the truncated proteins that lack ganglioside binding activity suggests that interaction of tetanus toxin with ganglioside is important for binding to neuronal cells.

Numerous studies have documented the binding of tetanus toxin to gangliosides both in vitro and in vivo, but it is not clear if these gangliosides represent functional receptors. Marxen et al. (17) reported that chromaffin cells are sensitive to tetanus toxin only after the addition of exogenous gangliosides. In another study (18), differentiation of PC12 cells with nerve growth factor led to an increase in tri- and tetrasialogangliosides that correlated with increased levels of tetanus toxin binding. It has also been reported, however, that the treatment of cells or neuronal membranes with proteases reduces tetanus toxin binding, suggesting that a protein component may be involved (23, 29). One model (18) proposes that tetanus toxin binds first to gangliosides and that this complex then interacts with a second receptor. A putative protein receptor in PC12 cell membranes has been identified based upon its ability to form a covalent complex with 125I-labeled tetanus toxin in the presence of a bifunctional cross-linker (30). Further analysis of Hc in addition to the identification of neuronal cell tetanus toxin receptors will help to characterize the process by which tetanus toxin binds to and intoxicates these cells.

Acknowledgments—We thank William Habig, Virginia Johnson, Scott Stibitz, and Luca Williamson for helpful discussions and Luca Williamson for preparation of spinal cord cell cultures.

REFERENCES
1. Eisel, U., Jurasech, W., Getzki, K., Henschen, A., Engels, J., Weller, U., Hudsell, M., Habermann, E., and Niemann, H. (1986) EMBO J. 5, 2495–2502.
2. Fairweather, N. F., and Lyness, V. A. (1986) Nucleic Acids Res. 14, 7809–7812.
3. Habermann, E., and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 193–197.
4. Niemann, H. (1991) in Sourcebook of Bacterial Protein Toxins (Alouf, J. E., and Freer, J. H., eds.) pp. 303–345. Academic, New York.
5. Helting, T. B., Parschat, S., and Engelhardt, H. (1987) J. Biol. Chem. 252, 1079–1075.
6. Matsuda, M., and Yoneda, M. (1974) Biochem. Biophys. Res. Commun. 57, 1257–1262.
7. Helting, T. B., and Zwisler, O. (1977) J. Biol. Chem. 252, 187–193.
8. Bittner, M. A., Habig, W. H., and Holz, R. W. (1989) J. Neurochem. 53, 379–385.
9. Ahnert-Hilger, G., Weller, U. Dauzenroth, M.-E., Habermann, E., and Gratzi, M. (1989) FEBS Lett. 242, 245–248.
10. Habermann, E., and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 193–197.
11. Niemann, H. (1991) in Sourcebook of Bacterial Protein Toxins (Alouf, J. E., and Freer, J. H., eds.) pp. 303–345. Academic, New York.
12. Horn, R., Dauzenroth, M.-E., Hudel, M., Weller, U., and Habermann, E. (1992) Toxicon 30, 63–76.
13. Morris, N. P., Consiglio, E., Kohn, L., and Habermann, E. (1990) J. Neurochem. 53, 379–385.
14. Weller, U., and Habig, W. H. (1987) Eur. J. Biochem. 164, 403–407.
15. Alouf, J. E., and Montecucco, C. (1991) Bacterial Toxins: Protein Toxins (Alouf, J. E., and Montecucco, C., eds.) pp. 164, 403–407.
16. Habermann, E., and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 193–197.
17. Laemmli, U. K. (1970) Science 175, 562–568.
18. Fairweather, N. F., and Lyness, V. A. (1986) Nucleic Acids Res. 14, 7809–7812.
19. Habermann, E., and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 193–197.
20. Matsuda, M., and Yoneda, M. (1974) Biochem. Biophys. Res. Commun. 57, 1257–1262.
21. Laemmli, U. K. (1970) Science 175, 562–568.
22. Fairweather, N. F., and Lyness, V. A. (1986) Nucleic Acids Res. 14, 7809–7812.
23. Habermann, E., and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 193–197.
24. Kenimer, J., and Habermann, E. (1990) J. Neurochem. 53, 379–385.
25. Kriegstein, K., Henschen, A., Weller, U., and Habermann, E. (1990) Eur. J. Biochem. 185, 39–45.
26. Sinha, Y., Klimpel, K. R., Quinn, C. P., Chaudhary, V. K., and Leppla, S. H. (1991) J. Biol. Chem. 266, 15490–15497.
27. Rolf, J. M., Gaudin, H. M., and Fidler, L. (1990) J. Biol. Chem. 265, 7331–7337.
28. Hans, P. C., Mietzner, T. A., Schoolnick, G. K., and McClane, B. A. (1991) J. Biol. Chem. 266, 11037–11043.
29. Varvogel, E., and Nathan, A. (1986) Eur. J. Biochem. 154, 403–407.
30. Schiano, G., Ferrari, G., Rossetto, O., and Montecucco, C. (1991) FEBS Lett. 290, 227–230.