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Disulfide–Immunogenicity Relationship of Botulinal Toxins (38002)

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The neurotoxic protein of nonproteolytic *Clostridium botulinum* type E is a progenitor toxin whose maximum toxicity is attained only when it is activated with an enzyme such as trypsin (1). Toxins of proteolytic types A, B, and F cultures are generally activated naturally.

Purified botulinal neurotoxins are proteins of mol wt 150,000–167,000. The naturally activated toxins are two-chain molecules made up of polypeptide units of which the larger is approximately twice the molecular weight of the smaller. Type E progenitor toxin is an "unnicked," single-chain protein. Although causal relationship is not yet proven, the toxicity increase resulting from its activation with trypsin is concomitant with cleavage of at least one peptide bond. The result is a molecule of two chains comparable to naturally activated toxins. Since demonstration of these subunits requires treatment with a disulfide-reducing agent, interchain disulfide(s) is important in holding these chains together in proper relationship (2).

A previous report showed that toxicity of the several tested types of botulinal toxin is critically dependent on the integrity of at least one disulfide bond (3). The work reported here is a related study on the possible importance of the S–S bond(s) to the antigenicity of botulinal toxins.

Materials and Methods. Type A toxin and activated type E toxin were taken as representatives of two-chain toxins; type E progenitor toxin was used as the single-chain toxin.

Highly purified type A and type E progenitor toxins (both mol wt 150,000) were similar to those used previously. Activated type E toxin was obtained by trypsinizing the progenitor toxin. Two equal portions were obtained from a sample. One was reduced with dithiothreitol (DTT) while the other was incubated without DTT. The method of obtaining the control and DTT-treated toxins was that described previously (3) except that buffer was made with mono- and dibasic sodium phosphate and did not have gelatin.

The incubated samples, minus a small volume saved for toxicity tests, were dialyzed at 37° against pH 7.4, M/15 sodium phosphate buffer containing (v/v) formalin at final 0.5%. Dialyzing buffer for the reduced toxin had 0.04 *M* DTT; control toxin was dialyzed against buffer without DTT. Changes to fresh solutions of these buffers were made daily for the first 4 days. Subsequent dialysis was with pH 6.4, M/15 sodium phosphate buffer containing formalin but not DTT. The treatment usually produced toxoids not lethal for mice (0.5 ml intraperitoneally) by the seventh day.

The toxoids were diluted as necessary and were homogenized in complete Freund adjuvant (Difco Laboratories, Detroit, MI) in a ratio (v/v) of 1.5 toxoid to 1.0 adjuvant. Each guinea pig (Hartley strain, 250–300 g body wt) of one group was injected subcutaneously with 1.5 ml of control antigen; individuals of a second group were immunized similarly with toxoid of DTT-treated toxin. Since control and test toxoids of a given toxin were prepared identically except for use of DTT, all animals of an experiment received the same amount of antigen protein.

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Tests for immune response were modifications of those used to determine the immunogenicity of toxoids prepared for use in humans (4). On the 28th day following the single immunizing dose, about 5 ml of blood from each guinea pig was obtained by cardiac puncture. On the 30th day post-immunization, two animals from each group were challenged with the homologous type of toxin (1.0 ml intraperitoneally). The following day a second pair of each group was challenged with a higher toxin dose if the original pair appeared normal and with the same or lesser dose if one or both were dead or appeared to be ill. The routine was followed on successive days until all animals were tested. A group of normal guinea pigs, not given immunizing treatment, was included for each experiment. Survival reported is based on observations of 4 days.

Sera from animals given the same antigen were pooled by combining equal volumes of the individual specimens. These pools were titrated for international units (IU) of antitoxin (4) using standard antitoxins obtained from the Center for Disease Control, Atlanta, GA.

Challenges of immunized guinea pigs were done, as appropriate, with crystalline type A toxin or crude type E toxin. The latter was the progenitor form obtained by extracting cells (5) of the Alaska E43 strain. This toxic preparation, which had been stored as a precipitate in 60% saturated $(NH_4)_2SO_4$, was dissolved in and dialyzed against M/15, pH 6.4 phosphate buffer containing 0.2% gelatin. Immediately before injection, the preparation was activated by adding an equal volume of 0.02% solution of crystalline trypsin (grade A, Calbiochem, Los Angeles, CA) and incubating at 37° for 30 min.

Toxicity is reported as ip LD_{50} for mice. For samples of high enough toxicity, these values were obtained by the intravenous assay (6). With toxin samples expected to have low toxicity, the intraperitoneal quantal assay (3) was used. The same assay procedure was used on samples being compared as to toxicity.

Results. Treatment of type A toxin with DTT decreased toxicity by 99.9%. When the control and reduced toxins were treated with formalin, the resulting toxoids differed significantly in their ability to stimulate antitoxic immune response in guinea pigs. In the original procedure (4), toxoids for use in humans are considered satisfactory if they can immunize guinea pigs to the level where half can survive a challenge of 100,000 mouse LD_{50}. Although the number of animals in the present test is limited, control animals survived challenges of up to 1×10^6 mouse LD_{50}. Guinea pigs immunized with toxoid of reduced toxin developed a tolerance to toxin only slightly higher than the natural resistance of normal animals (Table I). The latter value (about 10 mouse LD_{50}) is close to that reported by others (7).

Corresponding to their high resistance to direct challenge with toxin, animals immunized with the control type A toxoid had in their serum significant amounts of antitoxin. The serum pool made from blood of guinea pigs given toxoid of DTT-treated antigen did not have titratable antitoxin (Table I).

Tables II and III record results of similar experiments with type E progenitor toxin and activated type E toxin, respectively. The data justify the same conclusion as derived with type A antigens: reduction of disulfides of type E toxic preparations make the proteins poor immunizing antigens.

Resistance developed in response to toxoid of reduced type E progenitor toxin was higher than that obtained with the other reduced toxoids. This is probably because the particular reductive treatment was less effective than normally; whereas a 99+% loss of toxicity usually occurs (Ref. 3 and Tables I and III), in this particular instance the loss was 95%. Apparently, the slightly greater amount of unreduced antigen was able to stimulate an immune response which is better than normal for this category of toxoids.

When 50 μg of crystalline type A toxin was reacted against 10 IU of type A antitoxin (antiserum prepared with crystalline type A toxin) in Ouchterlony immunodif-

TABLE I. Comparison of Immunity Developed by Guinea Pigs in Response to Toxoid of Type A Botulinal Toxin (Control) vs Toxoid of Same Toxin Reduced with Dithiothreitol.

Immunizing toxoid[a]	Challenge dose LD_{50}/animal[b]	No. killed/No. tested	IU in serum pool[c]
Reduced	25	1/2	nil
	50	0/2	
	75	2/2	
	100	2/2	
Control	100,000	0/2	0.23
	250,000	0/2	
	500,000	0/2	
	1,000,000	0/2	

[a] Mouse ip LD_{50} before toxoiding: control = 6.7×10^6 LD_{50}/ml; reduced = 4.5×10^3 LD_{50}/ml. Immunizing dose of each toxoid: 15 μg antigen protein/animal.

[b] LD_{50} for mice; lethal dose for normal guinea pigs is about 10 mouse LD_{50}.

[c] International antitoxin units/ml in pool made by mixing equivolumes of serum from all animals of group.

fusion tests, the expected two immune precipitate lines developed. Only a single line was produced when a similar amount of DTT-treated crystalline toxin was used; this line gave a reaction of identity with the nontoxic β component isolated from the crystalline toxin (8). Failure to find the precipitate line corresponding to the neurotoxic component of the toxic crystalline moiety suggests that reduced type A neurotoxin loses its ability to precipitate with antitoxic antibody.

Discussion. It was shown previously that treatment of botulinal toxins with a disulfide reducing agent (DTT) destroyed almost all of the original toxicity (3). The present data show that a comparable S–S reductive treatment alters the proteins so that they become poor antigens for stimulating antitoxic immune response. As discussed relative to toxicity, the available data do not indicate whether or not the critical S–S bond(s) is, or includes, the interchain link of activated toxin or the intramolecular one(s) of type E progenitor toxin which becomes intermolecular when the progenitor is trypsinized.

Not shown here are the inconsistent results obtained in the early phase of this study; animals administered toxoids of DTT-treated toxins developed contradictory high or low immunity. The difficulty

TABLE II. Comparison of Immunity Developed by Guinea Pigs in Response to Toxoid of Type E Progenitor Toxin (Control) vs Toxoid of Same Toxin Reduced with Dithiothreitol.

Immunizing toxoid[a]	Challenge dose LD_{50}/animal[b]	No. killed/No. tested	IU in serum pool[c]
Reduced	100	0/2	trace
	500	0/2	
	1,000	1/2	
	5,000	2/2	
Control	50,000	0/2	0.28
	100,000	0/2	
	500,000	1/4	

[a] Mouse ip LD_{50} before toxoiding (not trypsinized): control = 1×10^4 LD_{50}/ml; reduced = 5×10^2 LD_{50}/ml. Immunizing dose of each toxoid: 20 μg antigen protein/animal.

[b] LD_{50} for mice; lethal dose for normal guinea pigs is about 25 mouse LD_{50}.

[c] See footnote *c* of Table I.

TABLE III. Comparison of Immunity Developed by Guinea Pigs in Response to Toxoid of Activated Type E Toxin (Control) vs Toxoid of Same Toxin Reduced with Dithiothreitol.

Immunizing toxoid[a]	Challenge dose LD_{50}/animal[b]	No. killed/No. tested	IU in serum pool[c]
Reduced	50	0/2	nil
	100	1/2	
	250	1/2	
	500	2/2	
Control	100,000	0/2	0.30
	250,000	0/2	
	500,000	0/2	
	800,000	1/2	

[a] Mouse ip LD_{50} before toxoiding: control = 8.8×10^5 LD_{50}/ml; reduced = 3×10^3 LD_{50}/ml. Immunizing dose of each toxoid: 20 μg antigen protein/animal.

[b,c] See footnotes *b* and *c* of Table II.

was resolved when the variable results were traced to the presence or absence of DTT during conversion of reduced toxin to toxoid. It appears that when the reducing agent is absent during its treatment with formalin, reduced toxin can regain a good part of its immunogenicity. This contrasts with our present inability to regenerate any meaningful degree of toxicity which is lost during reductive treatment of the toxins. The difference in recovery of the two activities is not surprising since, as shown by formalin-treated toxin being antigenic but nontoxic, the structural requirement for antigenicity is not as rigid as for toxicity.

Summary. Highly purified *Clostridium botulinum* types A and E (progenitor and trypsin-activated) toxins of mol wt 150,000 were treated with dithiothreitol, a disulfide-reducing agent. These reduced toxins and respective control (untreated) toxins were converted into toxoids with formalin and homogenized with complete Freund adjuvant. Guinea pigs immunized with control toxoids developed high resistance to challenge of homologous type of toxin; those immunized with toxoids of reduced toxins developed markedly lower immunity. Type A and E botulinal toxins have at least one disulfide bond whose integrity is important for these proteins to be effective immunizing agents.

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1. Duff, J. T., Wright, G. G., and Yarinsky, A. J., Bacteriol. **72**, 455 (1956).

2. DasGupta, B. R., and Sugiyama, H., Biochem. Biophys. Res. Commun. **48**, 108 (1972).

3. Sugiyama, H., DasGupta, B. R., and Yang, K. H., Proc. Soc. Exp. Biol. Med. **143**, 589 (1973).

4. Harrell, W. K., Green, J. H., and Winn, J. F., *in* "Botulism, Proceedings of a Symposium" (K. H. Lewis and K. Cassel, eds.), p. 165, Public Health Service Publication No. 999-FP-1 (1964).

5. Kitamura, M., Sakaguchi, S., and Sakaguchi, G., Biochim. Biophys. Acta **168**, 207 (1968).

6. Boroff, D. A., and Fleck, U., J. Bacteriol. **92**, 1580 (1966).

7. Cardella, M. A., *in* "Botulism, Proceedings of a Symposium" (K. H. Lewis and K. Cassel, eds.), p. 113. Public Health Service Publication No. 999-FP-1 (1964).

8. Boroff, D. A., Townend, R., Fleck, U., and DasGupta, B. R., J. Biol. Chem. **241**, 5165 (1966).

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