ANTIGENIC SUBSTRUCTURE OF TETANUS NEUROTOXIN

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SUMMARY. Immunochemical analyses were made on tetanus neurotoxin using horse antitoxin serum. Three distinct antigenic determinants were found in the neurotoxin molecule: one (α) in fragment α, released with fragment β by mild trypsin treatment of the toxin, and two (β-1 and β-2) in two subfragments (β-1 and β-2) of fragment β. Two fragments were obtained by mild treatment of the toxin with papain; two determinants (α and β-2) were found in one fragment and one determinant (β-1) in the other fragment. No anti-α antibody was found in human antitoxin serum. Later a fourth, "conformation dependent" antigenic determinant (designated γ) was found in the whole toxin molecule. The structure of tetanus neurotoxin was discussed.

In previous work we isolated and purified two, antigenically active complementary polypeptide fragments (fragment α, mol. wt. ca. 53,000 and fragment β, mol. wt. ca. 107,000 daltons) of tetanus neurotoxin (mol. wt. ca. 160,000 daltons) in sufficiently native states to be reconstituted into the whole toxin molecule (1, 2, 3). We also isolated a subfragment of fragment β by treatment with trypsin (EC 3.4.4.4) and two antigenically active components of the toxin by mild treatment with papain (EC 3.4.4.10).

This paper reports immunochemical analyses of the toxin employing purified preparations of these fragments and a subfragment. The antigenic structure of tetanus neurotoxin is discussed on the basis of the findings.

MATERIALS AND METHODS. Tetanus toxin was prepared and purified from bacterial extracts ("intracellular" toxin) and the complementary fragments α and β were separated and purified from mildly trypsinized (4) "intracellular" toxin, as described in our previous report (1). Fragment β-1 (a subfragment of fragment β) was prepared by treatment of fragment β with trypsin (trypsin to fragment β, 1:100, at 37 C for 2 hours) in 50 mM tris(hydroxymethyl)aminomethane [Tris]-0.6 M glycine, 1 mM EDTA, 2 M urea, 1 mM dithiothreitol(DTT)(pH 8.5); it was concentrated by equilibrium dialysis against 70% saturation (final) of ammonium sulfate in the same buffer and purified by gel filtration as described previously for the purification of fragments α and β (1). Mild papain treatment of the toxin (1.0-2.6 mg/ml) was performed in 0.1 M KNa phosphate buffer, pH 6.5 containing 0.7 mM cysteine, with a ratio of enzyme to toxin of 1:126, at 25 C for 60 min. The products were separated and purified by gel filtration on an Ultrogel AcA 44 column (1.5 by 90 cm) equilibrated with 50 mM Tris-0.6 M glycine buffer (pH 8.5) containing 1 mM EDTA. Horse antitoxin sera were gifts from the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. Human tetanus antitoxin sera, "Tetanobulin"

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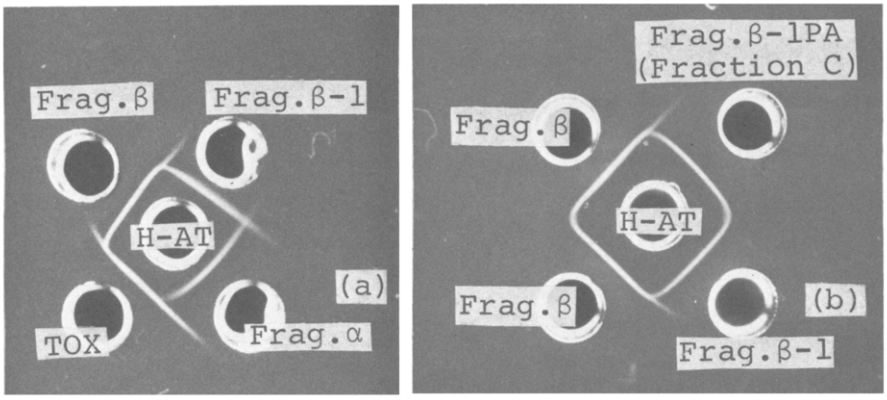

Fig. 1a. Immunodiffusion pattern of toxin, fragments α, β and β-1. H-AT, horse antitoxin (lot no. B215 450 U/ml); TOX, toxin (1.2 mg/ml); Frag., fragment; fragment α (0.3 mg/ml), fragment β (0.6 mg/ml), fragment β-1 (0.15 mg/ml).
Fig. 1b. Immunodiffusion pattern of fragments β, β-1 and β-1PA(Fraction C). H-AT, horse antitoxin (lot no. B139-P 400 U/ml); Frag., fragment; fragment α (0.8 mg/ml), fragment β-1 (0.34 mg/ml), fragment β-1PA (0.34 mg/ml).

were obtained from Midori-Juji Co., Osaka. Absorption of horse antitoxin serum was carried out by incubating the serum at 37 C for 1 hour and then at 4 C for 48 hours with fragment α, and then incubating the resulting supernatant with fragment β under suitable conditions to precipitate the corresponding antibodies completely; suitable conditions for this were established in preliminary quantitative precipitation reactions. Other analytical methods, including immunoelectrophoresis (3), sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis for molecular weight estimation, immunodiffusion, and estimation of protein (1), and the chemicals used (1, 3) were as described previously. Papain (30 U/ml, 10 mg/ml) was purchased from Boehringer Mannheim, Germany.

RESULTS AND DISCUSSIONS

Antigenic specificities of toxin fragments obtained by trypsin treatment of the toxin: The complementary polypeptide fragments α and β, isolated from the mildly trypsinized "intracellular" toxin, were antigenically different but they were both partially identical with whole toxin (1). Fragment α was very sensitive to trypsin, but when fragment β was further treated with trypsin in the presence of 2 M urea, it was gradually degraded to a trypsin-resistant core (mol. wt. ca. 50,000 daltons by SDS-gel electrophoresis). This material was purified to homogeneity by gel filtration. Immunodiffusion against horse antitoxin serum showed that antigenically it was partially identical with fragment β but distinct from fragment α (Fig. 1a). We designated this subfrag-

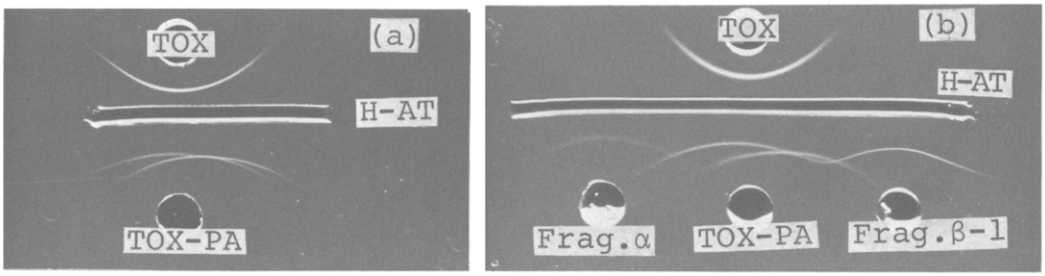

Fig. 2. Immunoelectrophoresis of untreated toxin and papain-treated toxin (mild conditions) (a, b). Electrophoresis was carried out at 4 C for 90 min at a constant voltage of 7.5 v/cm. The anode was on the right. (b), After electrophoresis, solutions of fragments α and β-1 were put into wells for immunodiffusion. H-AT, horse antitoxin (lot no. B215 450 U/ml); TOX, untreated toxin (1.0 mg/ml); TOX-PA, toxin after mild papain treatment (1.0 mg/ml); Frag., fragment; fragment α (0.38 mg/ml), fragment β-1 (0.3 mg/ml).

ment of fragment β, fragment β-1 and the rest of fragment β as "fragment β-2". Fragment β-1 had the same antigenic specificity as Fraction C (here named fragment β-1PA) prepared by prolonged papain digestion of the toxin as described by Helting and Zwisler (5) (Fig. 1b). The whole toxin molecule was relatively resistant to trypsin treatment, but fragment β-1 could be obtained from the whole toxin in low yield by proloned (37 C for 24 hours) trypsin treatment (enzyme to toxin, 1:100, at pH 8.0) in the absence of urea and in high yield by brief trypsin treatment (37 C for 1 hour) in the presence of 2 M urea.

Two antigenic components of "intracellular" toxin obtained by mild papain treatment and their antigenic specificities: When the toxin had been treated with papain (enzyme to toxin, 1:14) at 55 C for 4 hours as described by Helting and Zwisler (5), essentially a single antigenic component, fragment β-1PA remained. However, on milder treatment with papain (enzyme to toxin, 1:126) at 25 C for 60 min, the toxin dissociated into two antigenically active components with different electrophoretic mobilities, even in the absence of a denaturant such as urea (Fig. 2a): the component with lower mobility had the same antigenic specificity as fragment β-1, and the faster component had the same as that of fragment α and also another antigenic determinant distinct from that of fragment β-1 (Fig. 2b). On immunodiffusion against horse anti-

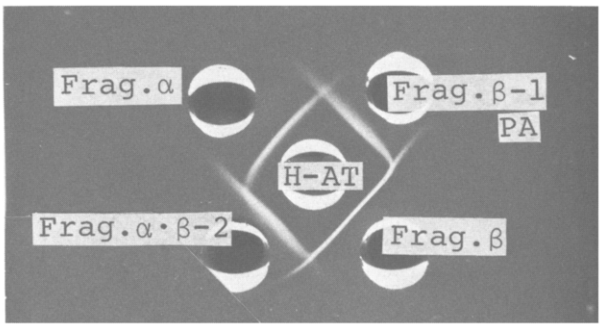

Fig. 3. Immunodiffusion pattern of fragments α, β and the components obtained by papain treatment (Frag.α·β-2 and Frag.β-1PA) against horse antitoxin. Frag., fragment; fragment α·β-2 complex (0.2 mg/ml) moved faster than fragment β-1PA (0.15 mg/ml). Other conditions were as described in the legend for Fig.1a.

toxin serum, the purified faster component formed a single precipitation band which fused with that of fragment α with spur formation and with that of fragment β with double spur formation (Fig. 3). Thus antigenically the faster component was composed of fragment α and fragment β-2 (fragment α·β-2).

Therefore, fragment β-2 must be situated between fragments α and β-1 in the whole toxin molecule. Moreover the two components obtained by papain treatment, fragments α·β-2 and β-1PA, must be less firmly associated togehter (by noncovalent bonds) than fragments α and β (1) (Fig. 4). On further reduction with 100 mM DTT and treatment with a denaturant such as SDS or urea (4 and 8 M urea for fragments α·β-2 and β-1PA respectively), fragments α·β-2 and β-1PA were both degraded to smaller pieces having no antigenic activity.

Absence of anti-α antibody in human antitoxin and presence of a "conformation dependent" antigenic determinant (designated γ) in the whole toxin molecule:

Fig. 5a shows results obtained on immunodiffusion analyses of human antitoxin serum with fragments α, β, β-1 and toxin, in comparison with results with horse antitoxin serum: in contrast to horse antitoxin serum, human antitoxin formed no precipitation band with fragment α. The single precipitation band formed between human antitoxin and toxin fused with that between human antitoxin and fragment β with clear spur formation (Fig. 5a). This spur must

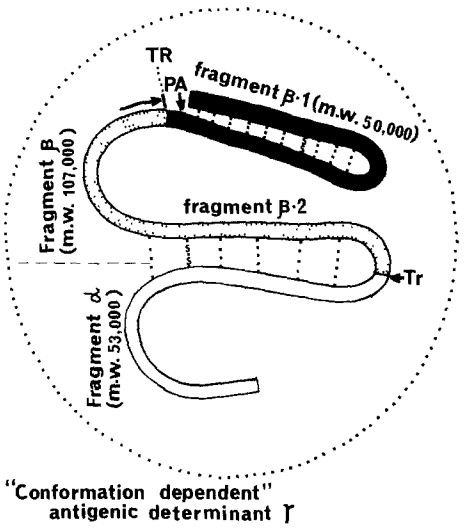

Fig. 4. Antigenic substructure of tetanus ("intracellular") neurotoxin. Tr, site(s) at which mild trypsin treatment (4) cleaves the single polypeptide chain (4) of the toxin. TR, point to which trypsin digestion (see Materials and Methods) of fragment β or of the toxin proceeds in the presence of 2 M urea. PA, site(s) at which mild papain treatment cleaves the peptide bond(s) or the point to which prolonged papain digestion (5) of the toxin proceeds. Dotted lines between the fragments indicate noncovalent bonds.

be due to antibody that reacted with neither fragment α nor fragment β, but with the whole toxin molecule, because this human antitoxin serum contained no detectable anti-α, the antibody against fragment α, the complementary fragment of fragment β. This was confirmed by the fact that after successive absorptions with fragment α and fragment β, horse antitoxin serum that could no longer precipitate either of these fragments still precipitated the whole toxin on an Ouchterlony plate (Fig. 5b). Similar results were obtained with several preparations of human and horse antitoxin sera tested. Fragments α.β-2 and β-1-PA could not precipitate all the antibodies which were reactive with the whole toxin. Therefore, there must be a fourth species of antibody which is directed against an antigenic determinant group (which we designated γ) present in the whole toxin molecule but not present or exposed in the isolated fragments.

Our results support the antigenic substructure of tetanus neurotoxin depicted in Fig. 4: tetanus toxin prepared from cell extracts is composed of

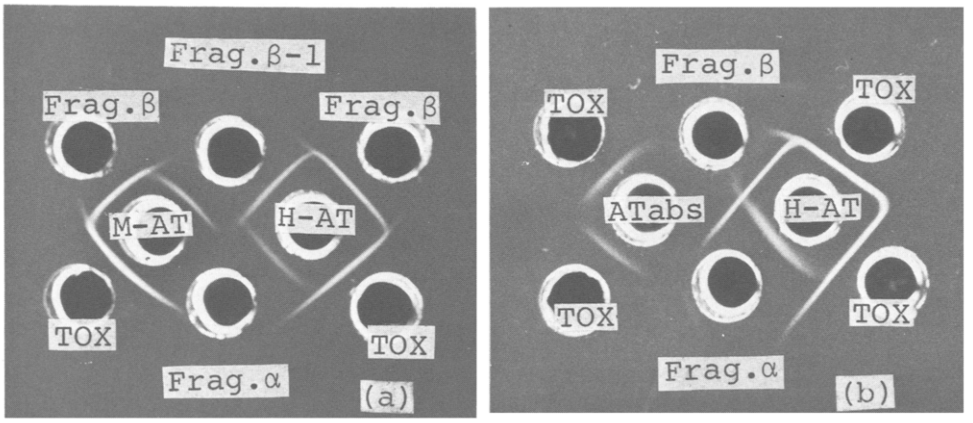

Fig. 5a. Immunodiffusion pattern of toxin, fragments α, β and β-1 against human and horse antitoxin sera. M-AT, human antitoxin (lot no. L73 200 U/ml); H-AT, horse antitoxin (lot no. B215 200 U/ml); TOX, toxin (0.6 mg/ml); Frag., fragment; fragment α (0.2 mg/ml), fragment β (0.43 mg/ml), fragment β-1 (0.26 mg/ml).
Fig. 5b. Immunodiffusion pattern of toxin, fragments α and β against absorbed and unabsorbed horse antitoxin. H-AT, horse antitoxin (lot no. B139-P 400 U/ml); ATabs, horse antitoxin absorbed with fragments α and β and concentrated to its original volume with minicon-A25 (Amicon Corp., Mass.). TOX, toxin (1.0 mg/ml); Frag., fragment; fragment α (0.38 mg/ml), fragment β (0.68 mg/ml).

three polypeptide portions, fragments α, β-2 and β-1 each with its own distinct antigenic determinant group. These fragments are bound together covalently by peptide bonds in this order, and fragments α and β are linked by a disulfide bridge. This scheme is in good agreement with the results very recently reported by Helting and Zwisler (6) on the structure of tetanus toxin prepared from culture filtrates. In addition, fragment α and β-2 or β are linked by noncovalent bonds that can be dissociated by treatment with 4 M urea or SDS, while the fragment β-1 portion of fragment β has an internal disulfide bridge (the other of the two disulfide bridges present in the whole molecule) and is relatively firmly bound within itself by noncovalent bonds that can only be dissociated by treatment with 8 M urea or SDS. This model explains the different degrees of sensitivity of the toxin, fragments α, β, β-2 and β-1 to trypsin and the processes of dissociation of the trypsinized toxin (4) and papain-treated toxin in the presence and absence of denaturants. The results show

that, besides these three distinct kinds of antigenic determinants (α, β-1 and β-2), tetanus toxin has a fourth determinant (γ) which is a "conformation dependent" antigenic determinant group. Thus the present results provide a molecular basis for the four kinds of antitoxic antibodies in tetanus antitoxin sera distinguished by Nagel and Cohen (7) using their "degraded" toxin preparations. The results of precise quantitative immunochemical analyses of various preparations of antitoxin sera will be published elesewhere.

REFERENCES

1. Matsuda, M., and Yoneda, M. (1975) Infect. Immun, 12, 1147-1153.
2. Matsuda, M., Hara, T., and Yoneda, M. (1975) Jpn. J. Med. Sci. Biol, 28, 326-328.
3. Matsuda, M., and Yoneda, M. (1976) Biochem. Biophys. Res. Commun, 68, 668-674.
4. Matsuda, M., and Yoneda, M. (1974) Biochem. Biophys. Res. Commun, 57, 1257-1262.
5. Helting, T., and Zwisler, O. (1974) Biochem. Biophys. Res. Commun, 57, 1263-1270.
6. Helting, T. B., and Zwisler, O. (1977) J. Biol. Chem, 252, 187-193.
7. Nagel, J., and Cohen, H. (1973) J. Immunol, 110, 1388-1395.