RECONSTITUTION OF TETANUS NEUROTOXIN FROM, TWO ANTIGENICALLY ACTIVE POLYPEPTIDE FRAGMENTS

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SUMMARY. Whole tetanus toxin indistinguishable from the undissociated toxin (molecular weight 160,000), in terms of molecular weight, electrophoretic behavior, antigenicity, and toxicity, was reconstituted. This was achieved by removal of urea and dithiothreitol (DTT) by dialysis from a mixture of freshly purified fragment α (molecular weight 53,000) and fragment β (molecular weight 107,000) in buffer containing 2 M urea and 1 mM DTT. The highest yield (almost 100%) of reconstituted toxin was obtained when the two isolated fragments were mixed in a molar ratio of 1:1. No toxin was reconstituted from either one of two fragments alone.

Recently we reported that tetanus neurotoxin (molecular weight, ca. 160,000), purified either from bacterial extracts ("intracellular" toxin, intact form) and mildly trypsinized, or from culture filtrates ("extracellular" toxin) was reversibly dissociated into two, antigenically distinct polypeptide chains, with molecular weights of approximately 53,000 (fragment α) and 107,000 (fragment β), by treatment with dithiothreitol (DTT) and urea under suitable conditions. We also reported a procedure for isolation and purification of these polypeptide fragments of the toxin in antigenically active forms (1).

This paper reports the reconstitution of tetanus neurotoxin from the two purified polypeptide fragments. Results showed that fragment α and β are functionally "complementary" in the whole toxin.

MATERIALS AND METHODS. Tetanus toxin was prepared and purified to homogeneity from bacterial extracts ("intracellular" toxin) and mildly trypsinized (2). Fragments α and β were separated and purified from urea-treated, DTT-reduced toxin by gel filtration on an Ultrogel AcA 44 column in the presence of urea and DTT, as described in our previous report (1).

The toxin was reconstituted from the purified fragments as follows: fresh preparations of purified fragment α (OD 280 nm = ca. 0.25 to 0.42) and purified fragment β (OD 280 nm = ca. 0.75 to 1.0) in 50 mM tris(hydroxymethyl)aminomethane (Tris)-0.6 M glycine, 1 mM EDTA, 2 M urea, 1 mM DTT (pH 8.5), were mixed in the desired molar ratio. The mixture was then dialyzed against 0.1 M sodium

potassium phosphate buffer, pH 7.5, at 0 C for 24 hours without stirring and for an additional 24 hours with gentle stirring to remove urea and DTT and permit disulfide bonds and noncovalent bonds to reform.

The toxicity of the reconstituted toxin was determined as the minimum lethal dose (MLD) in OF1 mice from the dose-survival time relationship, as described by Murata et al. (3).

Immunoelectrophoresis (4) was carried out using 1.5% agarose in 50 mM Tris-0.6 M glycine buffer, pH 8.5 containing 1 mM EDTA with the same buffer as the electrode buffer.

Other analytical methods including conventional-, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, molecular weight estimation, immunodiffusion and determination of protein, and the chemicals and horse antitoxin used were as described previously (1). Anti-fragment α rabbit serum was obtained by intramuscular injections (twice, 4 weeks apart) of formalin-treated (0.2%, at pH 7.8, 20 C for one week) fragment α (ca. 150 μg of protein per injection) in incomplete Freund's adjuvant.

RESULTS AND DISCUSSION

SDS-polyacrylamide gel electrophoresis of a dialyzed mixture of fragments α and β: On SDS-gel electrophoresis, the purified preparations of fragments α and β migrated individually as single protein components in positions corresponding to molecular weights approximately 53,000 and 107,000, respectively (Fig. 1A and B). However, when the two preparations freshly eluted from an Ultrogel column were mixed in a 1:1 molar ratio and dialyzed to remove urea and DTT and then examined on SDS-gel electrophoresis in the absence of a thiol reducing agent, the dialyzed material moved as essentially a single major protein component with the same molecular weight (ca. 160,000) (Fig. 1C) as that of undissociated toxin (Fig. 1D).

Immunoelectrophoresis and immunodiffusion of the dialyzed mixture of fragments α and β: We have shown that fragments α and β are antigenically distinct but that they are both partially identical with undissociated toxin (1). On immunoelectrophoresis employing horse antitoxin, a mixture of purified preparations of the two fragments (molar ratio, 1:1) in buffer containing 2 M urea and 1 mM DTT, behaved as two antigenic components with different mobilities (Fig. 2C). However, after removal of the urea and DTT by dialysis, the material behaved as a single antigenic component with the same mobility (Fig. 2B) as that of the undissociated toxin (Fig. 2A).

On immunodiffusion against horse antitoxin in Ouchterlony plates, the dialyzed material formed a single precipitation band which fused completely

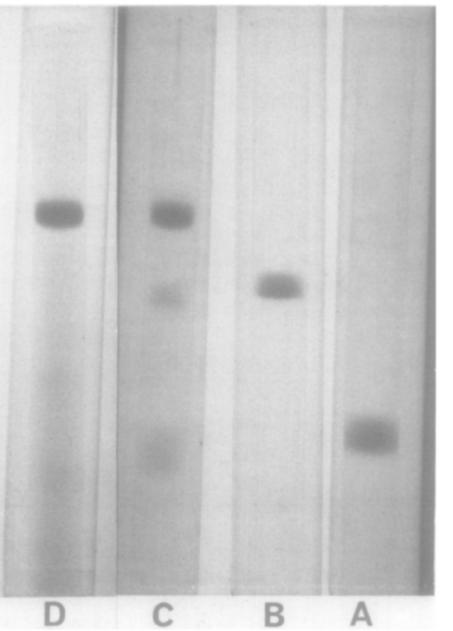

Fig. 1. SDS-polyacrylamide gel (5% gel) electrophoresis of purified fragment α (A), fragment β (B), a dialyzed mixture of fragments α and β (molar ratio, 1:1) (C) and undissociated toxin (D). Approximately 10 μg of protein were applied to the gels with application buffer in the absence of β-mercaptoethanol. Electrophoresis was performed at a constant current of 8 ma per gel for 6 hours. Migration was from top to bottom.

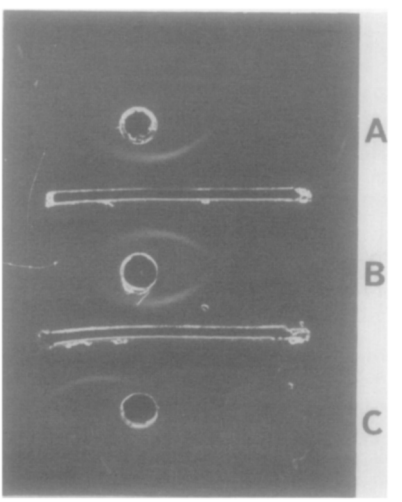

Fig. 2. Immunoelectrophoresis of a mixture of fragments α and β (molar ratio, 1:1), before (C) and after (B) dialysis, and of undissociated toxin (A). Approximately 20-μl samples containing approximately 700 μg of protein/ml were placed in each well. Electrophoresis was carried out at 4 C for 50 min at a constant voltage of 7.5 v/cm. The anode was on the right. Horse antitoxin (lot no. B215, 900 U/ml) was added to upper and lower troughs.

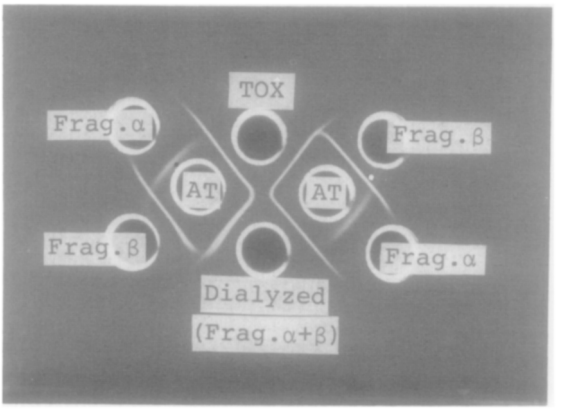

Fig. 3. Immunodiffusion patterns of toxin, fragment α, fragment β and a dialyzed mixture of fragments α and β (molar ratio, 1:1). TOX, undissociated toxin (1 mg/ml); AT, horse antitoxin (lot no. B215, 400 U/ml); Frag. α, fragment α (0.4 mg/ml); Frag. β, fragment β (1 mg/ml), and Dialyzed(Frag.α+β), a dialyzed mixture of fragments α and β (0.7 mg/ml).

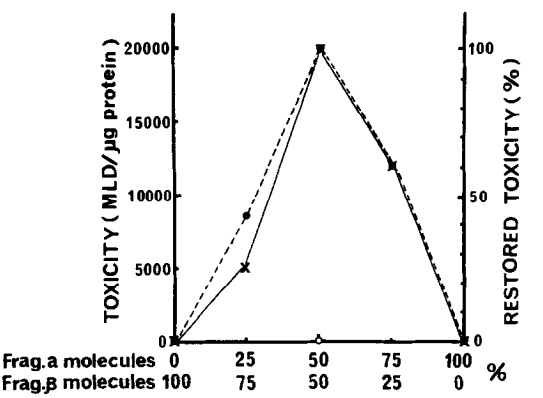

Fig. 4. Reconstitution of tetanus toxin from fragments α and β. Toxicities of a mixture of the fragments before (o) and after (x) removal of urea and DTT by dialysis. Right ordinate, percentage of toxicity restored; (•), theoretical values calculated assuming that all pairs of molecules of fragments α and β in the mixture reassembled into whole toxin molecules, irrespective of the presence of excess of one of the fragments. Abscissa, percentage of numbers of fragment α or β molecules in total numbers of fragment molecules in the mixture before dialysis.

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with the single precipitation band between antitoxin and undissociated toxin (Fig. 3). This dialyzed material formed single precipitation bands which fused with spur formation with the single precipitation bands between the antitoxin and fragments α and β, respectively (Fig. 3).

Toxicity of the dialyzed mixture of fragments α and β: As we reported previously (1) tetanus toxin looses its toxicity almost completely upon dissociation into fragments α and β. Purified preparations of fragment α showed little if any toxicity and no detectable peak of toxicity was associated with the protein peak of fragment α separated by gel filtration. The slight toxicity of purified preparations of fragment β (less than 1.2% of that of intact toxin on a protein basis) was completely neutralized by an amount of anti-fragment α rabbit serum only sufficient to neutralize a trace amount of intact toxin (0.01 toxin-neutralizing units of anti-fragment α/μg of fragment β). Therefore, we conclude that both fragment α and β are themselves intrinsically nontoxic and that the toxicity detected in purified preparations of these fragments is due to contaminating intact toxin. However, when essentially nontoxic, purified preparations of the two fragments were mixed in a molar ratio of 1:1 and then dialyzed to remove urea and DTT, and the dialyzed mixture was tested, it was found that the toxicity of the mixture had increased more than 190 times during dialysis (Fig. 4). The toxicity of the resulting material per μg of protein was about the same as that expected for undissociated toxin itself (approximately 2×10^4 MLD/μg).

On conventional gel electrophoresis, the dialyzed material gave essentially a single major protein band (Fig. 5B) in a position corresponding to that of the undissociated toxin (Fig. 5C). Plots of the toxicity extracted from slices of unstained gel showed that the restored toxicity was entirely associated with the single major protein band (Fig. 5A).

Similar experiments on the restoration of toxicity during dialysis of mixtures in various molar ratios of fragments α and β (Fig. 4) showed that a 1:1 molar ratio of the fragments gave the highest yield of toxicity and that no toxicity was restored on dialysis of fragment α or β alone.

These results indicate that the whole tetanus toxin molecule could be reconstituted from the two, nontoxic and antigenically distinct constituent polypeptide chains, fragment α and fragment β. This, together with our previous

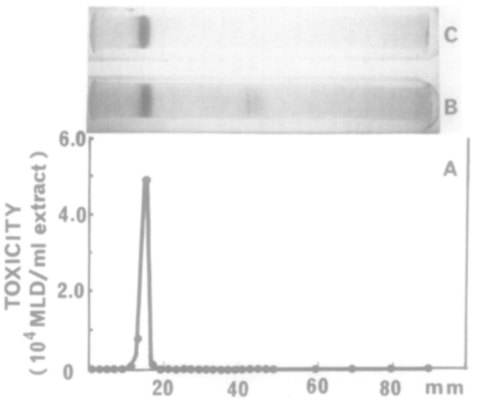

Fig. 5. Plot of the toxicity extracted from slices of unstained gel (A) and the electrophoretic pattern (B) of a dialyzed mixture of fragments α and β (molar ratio, 1:1) on conventional polyacrylamide gel (7.5% gel) electrophoresis (C), Electrophoretic pattern of undissociated toxin. Samples of approximately 10 μg of protein were applied for stained gels (B and C). A sample of approximately 20 μg of protein was applied to gel for the toxicity test (A). Toxicity was determined on extracts obtained from 2-mm slices of gel by extraction with 0.8 ml of phosphaphate buffered saline containing 0.2% gelatin at 4 C, overnight.

results (1, 2), provides definite evidence that either "extracellular" or mildly trypsinized "intracellular" tetanus neurotoxin is composed of two polypeptide chains, fragment α and β, linked at least by one disulfide bridge and associated by noncovalent bonds which are dissociated by detergents such as urea and SDS. The results also indicate that the fragments α and β isolated by our procedure are in sufficiently native forms to be able to reassemble in high yield into the fully functional, whole tetanus toxin molecule. Moreover these fragments are "complementary" to each other in the whole toxin in terms of molecular weight, antigenicity, and toxicity. Thus these two isolated fragments can be used for elucidation of the structure-function relationship of the tetanus toxin molecule. Experiments on the chemical and immunological properties of these fragments and their roles in the neurotoxic activity are in progress in our laboratory.

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REFERENCES

1. Matsuda, M., and Yoneda, M.(1975) Infect. Immun. 12, 1147-1153.
2. Matsuda, M., and Yoneda, M.(1975) Biochem. Biophys. Res. Commun. 57, 1257-1262.
3. Murata, R., Wada, E., and Kubota, K.(1956) Jpn. J. Bacteriol. 11, 797-802 (in Japanese).
4. Scheidegger, J. J.(1955) Int. Arch. Allergy Appl. Immunol. 7, 103-110.