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STUDIES ON THE ANTIBODY COMPOSITION AND NEUTRALIZING ACTIVITY OF TETANUS ANTITOXIN SERA FROM VARIOUS SPECIES OF ANIMALS IN RELATION TO THE ANTIGENIC SUBSTRUCTURE OF THE TETANUS TOXIN MOLECULE[1]

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SUMMARY On the basis of the antigenic substructure of tetanus neurotoxin, the antitoxin compositions of horse, rabbit and human tetanus antitoxin sera, in terms of their contents of antibodies against four antigenic determinant groups (α, β-1, β-2 and the "topographic" determinant group γ) so far known for the toxin were studied by quantitative precipitation reactions using purified toxin, complementary fragments α, β and fragment β-1 (a subfragment of fragment β) of the toxin. The antitoxin antibody composition varied slightly depending on the antiserum preparation. In addition, different patterns of antitoxin antibody composition and toxin-neutralizing ability, characteristic of horse, rabbit and man were found: horse antitoxin sera contained all four kinds of antibodies and horse anti-γ showed low toxin-neutralizing ability, while human antisera lacked anti-α and had anti-γ with high neutralizing activity but contained anti-β-1 with no detectable neutralizing activity. Rabbit sera showed an intermediate pattern between those of horse and human sera. In all antisera, antibodies against determinants on the isolated fragment β account for approximately 80–50 percent of the total precipitable antibodies and anti-β-2 antibody was invariably present. Immunodiffusion analyses showed that the antitoxin compositions of mouse and guinea pig antisera resembled those of human antisera. In mice, fragment β was almost as efficient as whole toxin toxoid in eliciting a protective immune response on an equal weight basis, whereas fragments β-1 and α were both relatively poor antigens.

INTRODUCTION

In previous communications (Matsuda and Yoneda, 1975; 1977) we reported the antigenic substructure of tetanus toxin (Fig. 1). Tetanus toxin (M_r ca. 150,000 daltons) prepared from cell extracts is composed of three polypeptide portions, each having M_r ca. 50,000

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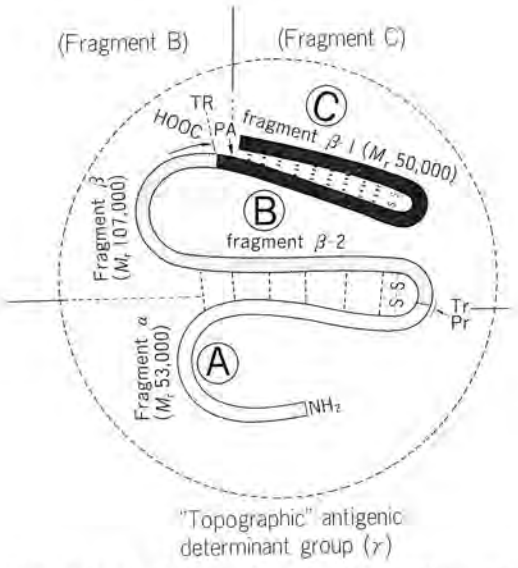


FIGURE 1. Antigenic substructure (tripartite model) of tetanus toxin.

" Intracellular " toxin (single polypeptide of M_r ca. 150,000 daltons) or " extracellular " toxin, which is composed of a light chain (fragment α, M_r ca. 50,000 daltons) and heavy chain (fragment β, M_r ca. 100,000 daltons) has four distinct antigenic determinant groups; three, α, β-2 and β-1 carried by fragment α, fragment β-2 and fragment β-1, respectively, and located in the toxin molecule in this order, and one additional so-called " topographic " antigenic determinant group, γ, which is detectable in the whole toxin molecule, but not in the isolated fragments. Tr, Pr, sites at which mild trypsin treatment and protease in the culture fluids nick the polypeptide chain. TR, point to which extensive trypsin digestion proceeds. PA, site(s) at which mild papain treatment cleaves the peptide bond(s) or the point(s) to which prolonged papain digestion of the toxin proceeds. S–S indicates a disulfide bridge and thin and thick dotted lines between fragments and within a fragment indicate noncovalent bonds that can be dissociated by 4 M urea or SDS, and by 8 M urea or SDS, respectively.

Designations of fragments in parentheses are according to the nomenclature of Helting et al. (1977a, 1977b, 1978). The new nomenclature for portions of the toxin molecule proposed by us according to the functions of these portions and to avoid confusion (Matsuda et al., 1982b) is shown in circles.

daltons. Fragments α, β-2 and β-1, each with its own distinct antigenic determinant group, are bound together covalently by peptide bonds in this order and linked by a disulfide bridge and noncovalent bonds between fragments α and β, while tetanus toxin prepared from culture filtrates has a nick in the polypeptide between fragments α and β. We have shown that, besides these three distinct kinds of antigenic determinant groups (α, β-2 and β-1), tetanus toxin has a fourth determinant group (γ) which is a " topographic " antigenic determinant group, in the sense that it is present in the whole toxin molecule but not present or not exposed in the isolated fragments (Matsuda and Yoneda, 1977).

In this study, on the basis of the above findings, employing purified preparations of toxin, fragments α, β and β-1, we analyzed the antibody composition and the toxin-neutralizing activity of tetanus antitoxin sera prepared in horses, rabbits, men, mice and guinea pigs, in terms of four kinds of antibodies directed against the four different antigenic determinant groups on the tetanus toxin molecule, by quantitative immunochemical study and immunodiffusion. We also examined the protection against tetanus toxin by active immunization of mice with these fragments.

MATERIALS AND METHODS

1. *Tetanus toxin and fragments of the toxin*

Tetanus toxin was prepared and purified from bacterial extracts (intracellular toxin) (Matsuda and Yoneda, 1974) and complementary fragments α (light chain) and β (heavy chain) were separated and purified from mildly trypsinized (Matsuda and Yoneda, 1974) intracellular toxin as described previously (Matsuda and Yoneda, 1975). Fragment β-1 was prepared as described in a previous report (Matsuda and Yoneda, 1977). The standard tetanus toxin (Lot TA-4A, 1 Test Dose for determination at $L_+/10$ level = 0.025 mg), used for challenge of the immunized mice and for assay of the toxin-neutralizing activity of the antisera, was a gift from the National Institute of Health, Tokyo.

2. *Tetanus antitoxin sera*

Horse antitoxin sera, including Lots B139, B215 and B324, were gifts from the Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University. Horse antitoxin serum (Lot A54, 5 U/ml), used as a standard in assay of toxin-neutralizing activity of antitoxin sera, was a gift from the National Institute of Health, Tokyo. Rabbit antitoxin sera were obtained by intramuscular injections (twice, at 100 Lf, 4 weeks between injections) of formalin-treated purified toxin, first in complete or incomplete Freund adjuvant and second in incomplete Freund adjuvant. Human tetanus antitoxins ("Tetanobulin") were purchased from Midori-Juji Co., Osaka. Mouse and guinea pig antitoxin sera were obtained by subcutaneous injections [twice, of 0.4 and 0.5 ml of aluminum-phosphate adsorbed toxoid (10 Lf/ml) into mice (strain ICR, 10 weeks old, female) and guinea pigs (strain Hartley, 6 months old, male, ca. 350 g) respectively, 4 weeks between injections].

3. *Quantitative precipitation reaction*

Increasing amounts of antigen were added to a series of tubes containing a constant amount of serum, equivalent to 20 units of antitoxin (0.1 ml in 0.1% NaN_3) and the total volume was made up to 0.35 ml with PBS. After 1 h at 37 C and 2 days at 4 C, the precipitates were centrifuged, washed three times with chilled PBS, drained and dissolved in 0.9 ml of 0.1 *N* NaOH and their OD at 280 nm was determined.

4. *Measurement of protein content*

The protein contents of the specific precipitates obtained by the quantitative precipitation reaction and of the antigens were measured by the method of Lowry et al. (1951).

5. *Assay of toxin-neutralizing activity*

The neutralizing activity of the antisera was titrated by the method described in Minimum Requirements for Biologic Products of Japan (1963), using standard toxin (Lot TA-4A) at a level of $L_+/10$ and antitoxin (Lot A54, 5 International Units/ml) provided by the National Institute of Health of Japan, Tokyo. OFl mice weighing 20–22 g were used.

6. *Tetanus toxoid and formalinization of the toxin and fragments of the toxin*

Tetanus toxoid adsorbed to aluminum phosphate employed for obtaining mouse and guinea pig antitoxin sera and as a reference of conventionally used toxoid in experiments on immunogenic activity was a product of Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. Formalin treatment of the purified toxin and the fragments was carried out after dialyzing the preparations against 0.067 *M* KNa phosphate buffer, pH 7.8, containing 0.025 *M* lysine under the conditions described in our previous report (Matsuda and Yoneda, 1976).

7. *Tests of immunogenic activity of toxin and fragments of tetanus toxin*

Mice (strain ICR, 5 weeks old, females, 18–20 g) were immunized with each formalin-treated fragment or the whole toxin toxid [extracellular (conventional) or intracellular toxin toxoid] by a single subcutaneous injection of 0.5 ml of five (or six) graded doses of each antigen without adjuvant and were challenged with 20 LD50 toxin 4 weeks later. The protective activity was evaluated using the score system described by Murata et al. (1961) by observing symptoms for 7 days.

8. *Analytical methods and chemicals*

Other analytical methods, including sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Matsuda and Yoneda, 1974) and immunodiffusion and the chemicals used (Matusda and Yoneda, 1975; 1976) were as described previously.

RESULTS

1. *Quantitative precipitation of antitoxins by toxin and fragments of the toxin*

Figure 2 shows, with dissociated toxin as a reference, the SDS-polyacrylamide gel electrophoretic patterns of the purified preparations of toxin and fragments α, β and β-1, which we employed in the quantitative analyses. Figure 3A shows their antigenically distinct relation against horse antitoxin serum (lower central well) on immunodiffusion. Fragments α and β, which are functionally complementary, showed distinct antigenicities, while both fragments showed partial identity with toxin. Fragment β-1 formed a line of partial identity with fragment β. Frag-

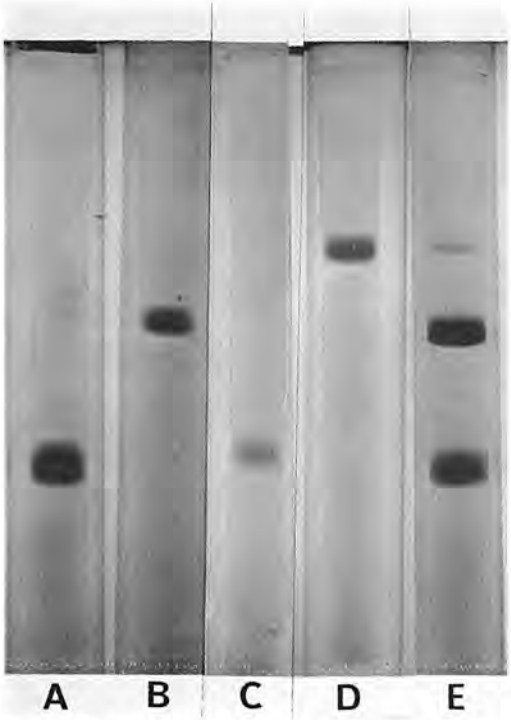
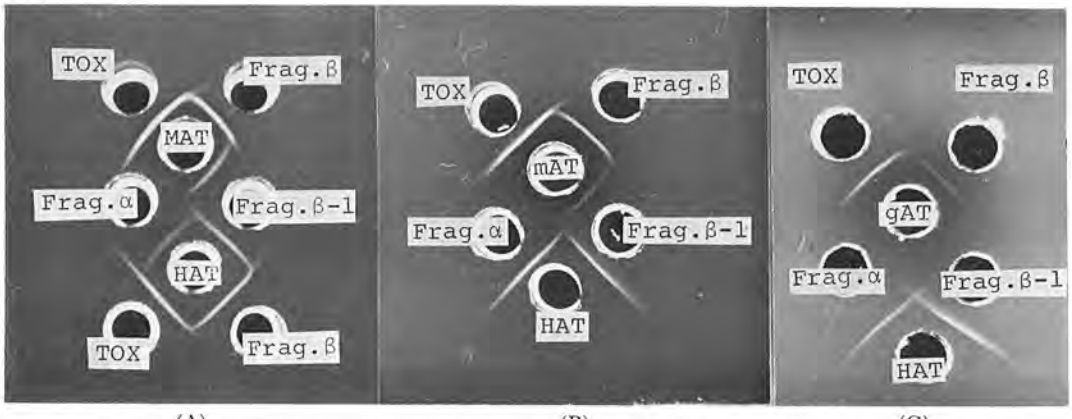


FIGURE 2. SDS-polyacrylamide gel electrophoretic patterns (5% gel) of purified preparations of tetanus intracellular toxin (D), fragment α (A), fragment β (B) and fragment β-1 (C) and of mildly trypsinized and dithiothreitol-reduced, dissociated toxin (E) as a reference.

ment α did not form a precipitation line against human antitoxin (Fig. 3A, upper central well).

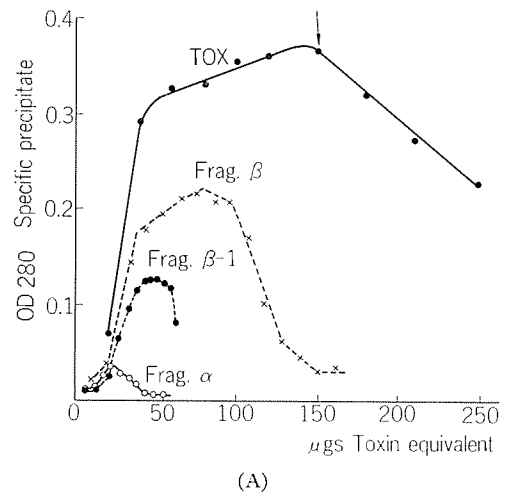
These differences in reactivity of antitoxins with various antigen preparations were studied in detail by quantitative precipitation using horse, rabbit and human antitoxins. Figure 4 shows representative plots of these quantitative reactions. The quantities of the four antigens added are expressed in micrograms of toxin equivalent. With horse antitoxin serum, toxin gave a flocculation type of curve having an equivalence zone that is characteristic of most horse antiprotein sera of high titer (Fig. 4A), while it gave typical precipitin curves having a sharp maximum with rabbit and human antitoxic sera. Analysis of the supernatants with toxicity showed that up to and including the point of maximum precipitation, all the toxicity was precipitated. As indicated by the arrows, excess toxicity was detected in the supernatants once the maximum was exceeded.

The amounts of antibodies directed against determinants on each fragment were estimated from the maximum precipitation. The amounts of antibodies against determinants β-2 and γ were calculated by subtracting anti-β-1 from anti-β and by subtracting anti-α plus anti-β from total antitoxin, respectively.

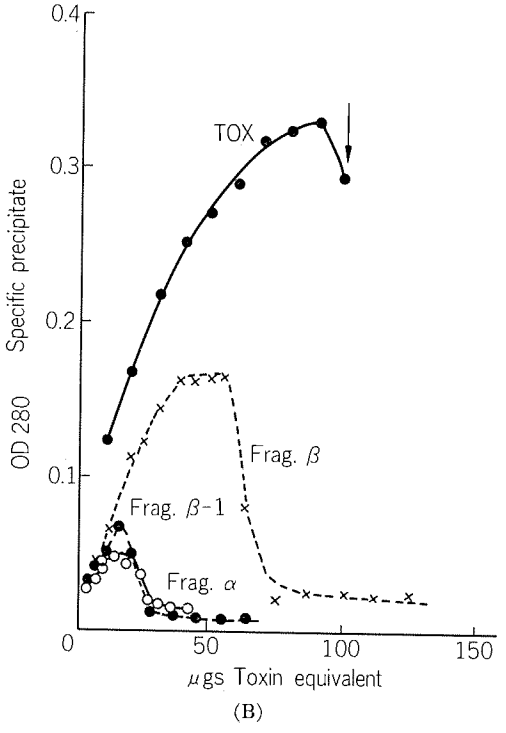


(A) (B) (C)

FIGURE 3. Immunodiffusion patterns of purified preparations of tetanus toxin (TOX, 300 μg/ml), fragment α (Frag. α, 70 μg/ml), fragment β (Frag. β, 280 μg/ml) and fragment β-1 (Frag. β-1, 60 μg/ml) against (A), horse antitoxin serum (HAT) and human antitoxin (MAT); (B), against mouse tetanus antitoxin serum (mAT), and (C), against guinea pig tetanus antitoxin serum (gAT).



(A)



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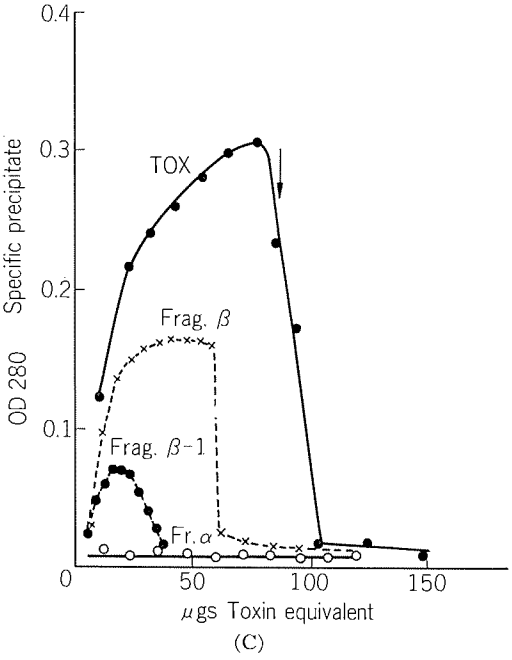

(C)

FIGURE 4. Quantitative precipitation of tetanus antitoxin sera with toxin and toxin fragments. (A), horse antitoxin; (B), rabbit antitoxin and (C), human antitoxin. TOX, toxin; Frag. α or Fr. α, fragment α; Frag. β, fragment β; Frag. β-1, fragment β-1. Amounts of antigen added are expressed in micrograms of toxin equivalent. Symbols: ●, toxin (TOX); ○, fragment α (Frag. α); ×, fragment β (Frag. β); ●---●, fragment β-1 (Frag. β-1).

The results are summarized in Table 1, column (P). The amounts are expressed as percentages of the amount of total antitoxin. Horse antitoxin sera invariably contained all four kinds of antibodies, though different antitoxin preparations varied in their proportions of antibody that could be precipitated by fragment α, β or β-1 (Table 1). On the other hand, none of the human antitoxins tested contained any detectable anti-fragment α (Fig. 3A, Fig. 4C, Table 1). In horse antitoxins, the percentage of anti-γ antibody varied remarkably depending on the particular preparation. Ten rabbits were immunized with purified whole toxin-toxoid in complete Freund adjuvant, followed 4 weeks later by a booster injection of toxoid, in complete Freund adjuvant in five rabbits, in incomplete Freund adjuvant in the other five. Antitoxin sera of two of five rabbits in each group con-

TABLE 1. *Antibody compositions of tetanus antitoxins from horse, rabbit and man*

Antitoxin		Amount of antibody precipitated[a] (P) (%)	Neutralizing activity associated with the antibody[b] (N) (%)	N/C[c]
Horse	(Lot B139)			
	Anti-α	10.9	12.5	1.15
	Anti-β	65.5	80.8	1.23
	Anti-β-1	42.4	47.5	1.12
	Anti-β-2[d]	23.1	33.3	1.14
	Anti-γ	23.6	6.7	0.28
	(Lot B215)			
	Anti-α	18.9	23.1	1.22
	Anti-β	77.5	81.5	1.05
	Anti-β-1	53.4	57.7	1.08
	Anti-β-2	24.1	23.8	0.99
	Anti-γ	3.6	<3.1	<0.86
Rabbit	(Lot I-3)			
	Anti-α	18.9	15	0.79
	Anti-β	48.1	55	1.14
	Anti-β-1	20.2	15	0.74
	Anti-β-2	27.9	40	1.43
	Anti-γ	32.8	30	0.91
	(Lot I-5)			
	Anti-α	0	—	—
	Anti-β	48.3	55	1.14
	Anti-β-1	23.8	10	0.42
	Anti-β-2	24.5	45	1.84
	Anti-γ	51.7	45	0.87
Human	(Lot L77)			
	Anti-α	0	—	—
	Anti-β	72.6	50	0.69
	Anti-β-1	32.8	<8.8	<0.27
	Anti-β-2	39.2	41.2–50	1.26
	Anti-γ	28.4	50	1.76
	Anti-whole toxin	100	100	1.0

[a] P, the amount of antibodies precipitable by each antigen preparation was determined by quantitative precipitation and expressed as a percentage of the total antibodies precipitable by the whole toxin molecule (anti-whole toxin).

[b] N, the toxin-neutralizing activity associated with the antibodies was calculated by determining the residual activity in the supernatant after precipitating the antibodies with each antigen preparation and subtracting the value from the activity before the precipitation. It is expressed as a percentage of the total toxin-neutralizing activity of the antitoxin serum (anti-whole toxin).

[c] N/P, ratio of the amount of precipitating antibodies to the toxin-neutralizing activity associated with the antibodies.

[d] The amount of anti-β-2 and its toxin-neutralizing activity were calculated by subtracting the values for anti-β-1 from those of anti-β.

[e] The amount of anti-γ and its toxin-neutralizing activity were calculated by subtracting the values of anti-α and anti-β from those of antibodies precipitable by the toxin (anti-whole toxin).

tained no anti-fragment α antibody. Examples of the two types of rabbit antisera are shown in Table 1. In contrast to antibodies directed against determinant groups γ and α, antibodies directed against determinant groups on fragment β, that is fragment β-2 and β-1 complex, were consistently present in tetanus antisera of all preparations from horse, rabbit and man, and fragment β precipitated approximately two-thirds to half the total antitoxins. From the percentages of the four kinds of antibodies, especially anti-α, and anti-γ antibodies in antitoxin sera, it appears that tetanus antitoxins show patterns of antibody composition characteristic of the particular animal species: the horse type is very different from the human type and the rabbit type is intermediate between these two types.

2. *Toxin-neutralizing activity of antitoxins*

To estimate the toxin-neutralizing potency of antibodies directed against the determinant groups on each fragment, we assayed the neutralizing activity remaining in the supernatant of the antiserum by the mouse method, after absorption with an amount of fragment just sufficient to result in maximum precipitation of the antitoxin. Loss of neutralizing activity in the antiserum was attributed to the antibodies precipitated by the fragment, and was expressed as a percentage of the total neutralizing activity of the antitoxin serum. The results of these experiments are summarized in column (N) of Table 1. Column N/P in Table 1 shows the toxin-neutralizing activity relative to the amount of precipitable antibody. Approximately 80–50 percent of the total neutralizing activity of the antisera was precipitated by fragment β (fragment β-2·β-1 compelex). In horse antitoxins, more than half the neutralizing activity was precipitated by fragment β-1, the anti-β-1 antibody of which is associated with higher neutralizing activity than anti-β-2, and only a small fraction of the neutralizing activity of which is attributable to anti-γ. In contrast, human antitoxins showed quite different characteristics: no detectable neutralizing activity was associated with anti-β-1, and high neutralizing activity was associated with anti-γ antibodies. The toxin-neutralizing activities of rabbit antitoxins showed roughly intermediate characteristics between those of horse and human antisera. In any case, anti-β-2 antibodies were constantly associated with high neutralizing activity, except in one horse antiserum Lot B215, irrespective of the species of animal from which the antiserum was obtained.

3. *Comparison by immunodiffusion analysis with purified toxin and its fragments, of tetanus antitoxin sera from mouse and guinea pig with those of human and horse*

Figure 3B shows results on immunodiffusion analysis of mouse antitoxin serum. The pattern shows the absence of anti-α antibody and the presence of anti-β-1 and anti-fragment β and by the spur formation between the bands of fragment β-1 and fragment β that is a complex of fragment β-2 and β-1, the presence of anti-β-2 antibodies. Spur formation between the precipitation bands of toxin and fragment β shows the presence of anti-γ antibody, because the mouse antiserum lacks anti-α antibody, which is directed against fragment α that is complementary to fragment β. The immunodiffusion pattern in Fig. 3 C with guinea pig antiserum, again indicates the absence of anti-α antibody. Anti-β-1 antibody was also undetectable and so the reaction with fragment β is that against anti-β-2 antibody. Therefore, spur formation between the bands of toxin and fragment β shows the presence of anti-γ antibody in guinea pig antiserum. Similar results were obtained with different preparations of antitoxin sera from mice and guinea pigs. Figure 3A shows the immunodiffusion pattern of human and horse antitoxin sera as a control.

4. *Immunogenic activities of fragments of tetanus toxin*

We first immunized groups of mice with

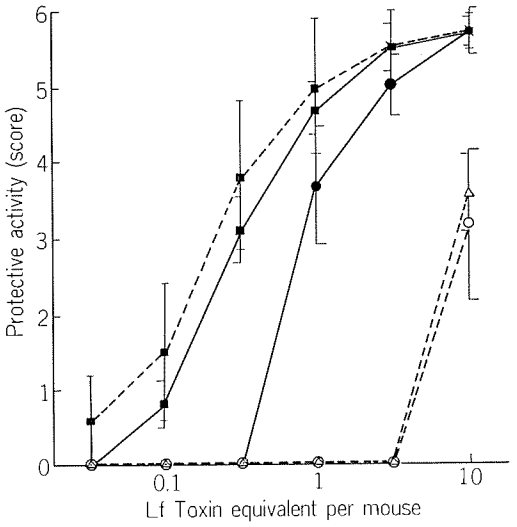

FIGURE 5. Dose-response curves in mice (strain ICR) to tetanus toxoid (extracellular toxin-toxoid, ■—■; intracellular toxin-toxoid, ■---■) and formalinized fragments, Frag. α (△---△), Frag. β (●—●) and Frag. β-1 (○---○) of tetanus toxin. Groups of 10 mice were immunized with five (or six) graded doses of the antigens (single subcutaneous injection in 0.5 ml, without adjuvant) and challenged 4 weeks later with 20 LD50 of toxin (0.5 ml subcutaneous injection) at 5 weeks of age (18–20 g, female). Protective activity was evaluated by the score method (Murata et al., 1961): score 0, death within two days; 1, death on day 3; death in 4–7 days or survival with severe tetanus; 4, survival with slight tetanus, and 6, no symptom. Observations were made for 7 days. The doses of antigens used for immunization are expressed in Lf units equivalent to the whole toxin. Values are means±S.E. for 6 mice.

toxoid derived from whole toxin or the formalin-treated fragment and then challenged them with toxin and evaluated the immunogenic activity against tetanus toxin using the score system (Murata et al. 1961) by observing symptoms for 7 days. Figure 5 shows the dose-response curves obtained in representative experiments. Fragments α and β-1 both showed roughly one-thirtieth as much protective activity as the whole toxin toxioid. Fragment β, which is composed of fragments β-2 and β-1 and is complementary to fragment α, was about one-third to half effective as the whole toxin toxioid on a molar basis, and so had roughly similar potency (50–100 percent) to the whole toxin toxiod on a weight basis.

DISCUSSION

The multiplicity of the antigenic determinants of tetanus toxin and the heterogeneity of tetanus antitoxins were first shown by Turpin and Raynaud (1959). They found that purified tetanus toxin after digestion with trypsin gave two distinct lines with most antitoxin sera on immunodiffusion. They considered that antibodies directed against only one of these determinants were responsible for neutralizing the toxicity of the tetanus toxin molecule. By freezing crude tetanus toxin, Peetoom and van der Veer (1967) obtained a degraded product that was devoid of toxicity and showed partial identity with toxin on immunodiffusion. Using this degraded tetanus toxin, Cohen et al. (1970) isolated two neutralizing antibodies from tetanus antitoxin. Subsequently, Nagel and Cohen (1973) extended these studies and demonstrated by successive absorption using three kinds of antigens -[1], -[2] and -[3], prepared from spontaneously degraded toxin, that tetanus antitoxic sera contain at least four antitoxins (Cohen et al., 1970; Nagel and Cohen, 1973). However, the structural relations of these degraded antigens to the tetanus toxin molecule are unknown.

We have elucidated the antigenic substructure of the tetanus toxin molecule (Matsuda and Yoneda, 1977). Using purified complementary toxin fragments that were sufficiently native to be reconstituted into the whole toxin molecule almost completely (Matsuda and Yoneda, 1976) and a subfragment of one of the complementary fragments, we demonstrated the presence in horse and rabbit tetanus antitoxic sera of at least four distinct antibodies each having toxin-neutralizing

activity. Although it is not known whether these four kinds of tetanus antitoxins are identical with those reported by Nagel and Cohen (1976), the present results provide exact information on the antibodies directed against four kinds of antigenic determinant groups whose locations in the tetanus toxin molecule were well defined: tetanus antitoxin sera contain three kinds of antibodies directed against three kinds of determinant groups (α, β-2, β-1) carried by three portions [fragment α, β-2 and β-1: fragment α and β-1 were reported to be N-terminal and C-terminal portions, respectively, of the toxin (Neubauer and Helting, 1979)] of tetanus toxin, and an additional antibody directed against a fourth "topographic" antigenic determinant group (γ), which is not detected in any of isolated fragments but is detected in the whole toxin molecule.

The above results of quantitative immunochemical analyses of the antibody composition of various preparations of antitoxin sera revealed heterogeneity in antibody composition, in terms of the four kinds of antitoxin antibodies (anti-α, anti-β-2, anti-β-1, anti-γ) in tetanus antitoxin sera not only in different animal species, but also in antiserum preparations derived from the same animal species. However, there appeared to be distinct patterns of antibody composition characteristic of particular animal species. For instance, anti-α antibody was detectable in antitoxic sera from horse, but not man. Rabbit antitoxin sera appeared to show an intermediate pattern between those of horse and human, anti-α antibody being detectable in only some rabbit antisera. Mouse and guinea pig antisera also showed a similar pattern to that of human antisera.

The fact that anti-α antibody was undetectable in human antisera may be correlated with the absence of anti-[1] antibodies in human antiserum reported by Nagel and Cohen (1973), and suggests the identity of anti-α antibody with the anti-[1] antibody that they reported. It is unlikely that the kinds of adjuvants used significantly affected the antibody patterns characteristic of different animal species and the heterogeneity in preparations. A horse immunized with complete Freund adjuvant and aluminum phosphate adjuvant (Lot B139) and one immunized with aluminum phosphate adjuvant (Lot B324) gave quite similar patterns of antitoxin antibody compositon. Some rabbits (two of five in each group) had no detectable anti-α antibodies irrespective of the kind of adjuvant (Freund incomplete or complete) with which they were immunized. However, precise studies are required on the effect of adjuvant on the antibody composition.

Discrepancies have been reported on the titers of tetanus antitoxin sera obtained using different antisera as references or standards (Yamamoto et al. 1970). The present results show that these discrepancies can be explained by heterogeneity in the composition of tetanus antitoxin and indicate the importance of obtaining information on the exact antibody composition of tetanus antitoxin sera used as references or standards.

The facts that, irrespective of the different patterns of antibody composition, the anti-β-2 antibody showed high toxin-neutralizing activity and was invariably present in antisera from all animal species tested seem to indicate that the β-2 portion of the tetanus toxin molecule is important in tetanus. In fact, on challenge with toxin in mice, fragment β, which is a complex of fragment β-2 and β-1, showed the highest immunogenic activity of the toxin fragments, being roughly comparable to that of the whole toxin toxoid.

Recently Helting and Zwisler (1978) reported extremely low, but nonspastic toxicity in one of the fragments from papain-digested toxin, fragment B, which corresponds to our papain fragment, fragment $\alpha\cdot\beta$-2 complex. Very recently we found acute botulinum-like toxicity on intravenous injection of tetanus toxin (Matsuda et al., 1982a) and reported that the acute toxicity was due to the fragment $\alpha\cdot\beta$-2 complex (Matsuda et al., 1982b). At

the meeting in 1978, at which we reported an outline of this work, Helting and Nau reported that protection against tetanus by active immunization with their fragment B (which corresponds to our fragment $\alpha \cdot \beta$-2) was as efficient as that with tetanus toxoid, whereas immunization with fragment C (which corresponds to our fragment β-1) was less efficient in guinea pigs but approached the protective capacity of tetanus toxoid in mice (Summaries of the Presentations, 5th International Conference of Tetanus, Ronneby, Sweden, June 18–23, 1978). These findings again indicate the important role of fragment β-2 in tetanus, because as we described above, no anti-α antibody could be detected in human, mouse or guinea pig antisera by the quantitative precipitation reaction or immunodiffusion and only very weak protective responses were elicited in mice by active immunization with both fragments α and β-1. For determination of the activity of fragment β-2, attempts to isolate fragment β-2 are in progress in our laboratory. Since purified preparations of fragment α showed no contamination with toxin in toxicity tests, the protection of mice immunized with fragment α against tetanus toxin is considered to be due to antibodies directed against fragment α. For detection of very low titers of anti-α antibody, a more sensitive method is needed than those employed in this study. We could not detect any anti-α antibody in human tetanus antitoxins by precipitation reaction. The possibility that anti-α antibody exists in a univalent form still remains to be investigated. The fourth "topographic" antigenic determinant group γ was detected by precipitation reactions in the supernatant after successive precipitations of the antitoxin serum with fragments. Thus it is possible that the supernatant contains antibodies against single determinants belonging to each of the α, β-2 and/or β-1 portions of the toxin molecule. This possibility must be examined by affiinity chromatographies using each fragment as a ligand.

The present results demonstrating the resemblance of the antibody compositions of human, mouse and guinea pig antisera indicate that mice can be used in place of guinea pigs, which are more expensive but have conventionally been used in routine potency assay of tetanus toxoid.

Recently monoclonal antibodies against tetanus toxin have been reported (Mizuguchi et al., 1982; Gigliotti and Insel, 1982). All the antibodies described above were studied by the precipitation reaction, so, exactly speaking, they are each groups of antibodies directed against a group of determinants on each fragment. For precise characterization of the antibodies against tetanus, monoclonal antibodies against tetanus toxin and its fragments are being isolated in our laboratory.

ACKNOWLEDGMENT

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