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# Cross-reactivity of Monoclonal Antibodies against *Clostridium perfringens* Θ Toxin with Streptolysin O

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**Abstract.** Six monoclonal antibodies to Θ toxin of *Clostridium perfringens* were characterized. Four antibodies, 1C3, 2D4, 1B9, and 3F11, were nonneutralizing for Θ toxin and were non-cross-reacting with streptolysin O (SLO). The other two antibodies, 3H10 and 2C5, were cross-binding and cross-neutralizing with SLO. Neutralizing activity of 3H10 was higher than that of 2C5 on the basis of the binding activity with Θ toxin and SLO. Both antibodies could inhibit hemolysis even after binding of the toxins to sheep red blood cells and inhibited cardiotoxicity of the toxins in cultured heart cells.

Thiol-activated cytolytic toxins such as streptolysin O (SLO), perfringolysin O (Θ toxin), and tetanolysin are known as a group of bacterial protein toxins with common biological and physical properties [1, 2, 3, 19]. They appear to be antigenically related because of the cross-reactivity of their antisera; cross-neutralization by the antibodies against Θ toxin, SLO, and other toxins has been reported [3, 4]. By the immunodiffusion method [4, 18] with horse antisera, it was suggested that the toxins are not antigenically identical, but share several common antigenic structures. These cross-reactivities, however, have been observed only in hyperimmune horse antisera and not in human or rabbit sera [4, 10]. The cross-reactivity of ten anti-SLO human sera with monoclonal hypergammaglobulinemia was studied by use of pneumolysin, tetanolysin, and Θ toxin as well as SLO. With one exception, there was no cross-neutralization in the 30 possible combinations involving the first three antigens [10]. These findings indicate that the antibodies were reacting with antigenic sites not common to each toxin. In spite of the observations cited above, it is still unclear whether the common antigenic sites shared by the cytolytic toxins are sites related to their biological activity. Since monoclonal antibodies appear to be a powerful tool for resolution of this question, we attempted to prepare mouse monoclonal antibodies against Θ toxin. In this study, two neutralizing and four nonneutralizing antibodies were characterized for cross-reactivity between Θ toxin and SLO, and for the binding site on the toxins. Among the monoclonal antibodies obtained, only those with Θ-toxin-neutralizing capacity were cross-reactive and cross-neutralizing with SLO. None reacting with tetanolysin was found in the cultures of any of the hybridomas obtained.

## Materials and Methods

**Toxins and toxoids.** Theta toxin (Θ) was purified from culture supernatant of *Clostridium perfringens* strain-BP6K as described previously [21]. As immunogen, the toxin (400 μg/ml) was dialyzed against 1/60 *M* phosphate-buffered saline, pH 7.0, containing 0.3% formalin and 0.05 *M* L-lysine for one day to reduce the lethal toxicity. Streptolysin O (SLO) was partially purified from culture supernatant of group-A *Streptococcus pyogenes* strain D58 by fractionation with ammonium sulfate and successive chromatography on DEAE-cellulose and hydroxylapatite. Tetanolysin was partially purified from culture supernatant of *Clostridium tetani* strain A47. Pass-through fractions from the DE52 column treated under the same conditions as for purification of tetanus toxin in the previous paper [15] were pooled and used. Pertussis toxoid was prepared as reported previously [14, 16].

**Preparation of hybridomas.** A BALB/c mouse (Charles River Japan, Kanagawa, Japan) was primed with an intraperitoneal injection of alum-precipitated Θ toxoid (100 μg protein/mouse) supplemented with pertussis toxoid (12.5 μg protein/mouse). Seven weeks later, Θ toxoid (80 μg/mouse) was given intravenously without adjuvant. Three days after the booster injection, the spleen of the immune mouse was removed for use in hybridoma production. The protocol for the fusion and selection of antibody-producing hybrid cell lines used in this study was as outlined by Oi and Herzenberg [12]. Spleen cells were fused to SP2/0-Ag14 myeloma cells (kindly donated by Dr. K. Ozato, NCI, NIH) at a ratio of 2:1 with use of 50% polyethylene glycol

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Table 1. Expected combination of reactivity of anti-θ monoclonal antibodies to θ toxin and SLO

| Type | θ | | SLO | |
|---|---|---|---|---|
| | B[a] | N[b] | B[a] | N[b] |
| 1 | + | − | − | − |
| 2 | + | − | + | − |
| 3 | + | − | + | + |
| 4 | + | + | − | − |
| 5 | + | + | + | − |
| 6 | + | + | + | + |

[a] Binding ability to θ toxin or SLO by ELISA.
[b] Neuralizing ability on hemolysis with θ toxin or SLO.

4000 (Merck, Darmstadt, FRG) and were cultured in four 96-well culture plates (Coster). After selection of hybrid cells with hypoxanthine–aminopterin–thymidine medium [12], the presence of the anti-Θ-toxin antibodies in the culture fluids was tested by the enzyme-linked immunosorbent assay (ELISA) using Θ-toxin-coated microplates, and by antihemolytic activity against Θ toxin. Out of 96 growing hybrid cell cultures, 78 were antibody-positive in ELISA, and about half of them contained neutralizing antibody.

**Selection of specific antibody-producing hybridomas.** Since hybridomas producing antibodies reacting with tetanolysin were not detected in the culture at the screening step, selection of the six types of hybridoma producing the antibodies shown in Table 1 was attempted using Θ toxin and SLO. Cloning was performed by limiting dilution [12]. The clone and its product, monoclonal antibody, were given the same name. At the first cloning, three clones—1C3 (type 1), 2D4 (type 1), and 3H10 (type 6)—were obtained, and at the second cloning 1B9 (type 1), 2C5 (type 6), and 3F11 (type 1) were obtained. No clone of type 2, 3, 4, or 5 was obtained.

**Preparation of monoclonal antibodies.** Culture fluid of each hybridoma was concentrated and tested for the isotype of the monoclonal antibody by the double immunodiffusion test with use of commercially available anti-mouse Igs (Bionetics, Inc., Kensington, MD). All of them were IgG1. To obtain ascites fluid with a high titer of the antibodies, the cells cloned at least twice were propagated and injected into BALB/c mice primed with pristane (2,6,10,14-tetramethyl pentadecane, Aldrich Chemical Company, Inc.). The antibodies from the culture fluids (CF) and the ascites fluid (AF) were purified by affinity chromatography with protein-A–Sepharose (Pharmacia Fine Chemicals) [6] and Θ toxin-combined CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) (Θ-Sepharose) [7].

**Assay of binding activity of the antibodies to the cytolytic toxins.** Binding activity of the antibodies to the cytolytic toxins was measured by ELISA, which was performed essentially according to the method reported previously [14, 20]. Polystyrene microplates were coated with Θ toxin (1 μg/ml) or SLO (5 μg/ml) for assays of binding to Θ toxin or SLO respectively. Antibodies bound to the antigens were allowed to react with alkaline phosphatase–anti-mouse IgG conjugate (Tago Inc.) and were detected by addition of the substrate, *p*-nitrophenylphosphate. By measurement of absorbance at 400 nm, dose–response curves were drawn on log–log graph paper as described in a previous paper [14]. Antibody titer on ELISA (ELISA U/ml) was estimated from the curves by the parallel line assay method by use of a reference antibody and was expressed as a relative titer. The reference antibody was anti-Θ mouse IgG, which was purified by affinity chromatography of Θ-sepharose from the sera of mice immunized with purified Θ toxoid, and was assigned one ELISA U/ml arbitrarily. Although it is unknown how much antibody reacting with SLO was contained in the reference antibody, the reactivity of the reference antibody with SLO was about 5% of that with Θ toxin by ELISA. To analyze the recognition site of the antibodies on Θ toxin, binding activity of the antibodies to the Θ toxin, which was specifically oriented by binding to sheep red blood cell (SRBC) membrane, was measured by the ELISA method; microplates were coated with the SRBC-membrane fraction [21] dissolved in the coating buffer (20 μg/ml), and then Θ toxin in PBS (1 μg/ml) was allowed to react with the immobilized membrane to obtain the same orientation of the toxin (SRBCM-Θ). Theta toxins modified with DTNB (5,5′-dithiobis[2-nitrobenzoic acid]) [5] or BIPM (N-[*p*-(2-benzimidazolyl)phenyl] maleimide) [17] were also used for coating of the microplate.

**Assay of antihemolytic activity of the antibodies.** Hemolytic activity of the toxins was measured essentially as described previously [21] and was expressed in terms of the reciprocal of the highest dilution showing complete hemolysis ($HD_{100}$). Fifty percent hemolysis ($HD_{50}$) was determined by measuring the absorbance at 550 nm of the supernatant solution of the reaction mixture.

Specific activity of Θ toxin and SLO was 200,000 $HD_{100}$/mg and 16,000 $HD_{100}$/mg, respectively. For the neutralization test, the test toxin (50 μl) diluted with PBS containing 0.2% gelatin to 2 $HD_{100}$/ml was mixed with antibodies (50 μl) diluted serially twofold in a microplate (V bottom, 96 wells, Limbro). After the reaction proceeded for 30 min at room temperature or 0°C, 1% SRBC suspension (100 μl) was added to each well. The plate was let stand for 30 min at 37°C and was centrifuged at 4°C for measurement of the absorbance of the supernatant. A point corresponding to one $HD_{50}$ of a residual toxin was determined from the absorbance at 400 nm, and neutralization activity was expressed by the reciprocal of the dilution of the antibodies at that point.

## Results

**Binding and neutralizing activity of the monoclonal antibodies to Θ toxin and SLO.** Binding activities of three monoclonal antibodies, 1C3, 2D4, and 3H10, to Θ toxins which were modified chemically with DTNB or BIPM, or with the cell-binding site masked by the SRBC membrane (SRBCM-Θ) as well as to a native Θ toxin, were measured (Table 2). Two antibodies, 1C3 and 2D4 with no toxin-neutralizing activity, bound to all four forms of toxin, but showed a slight difference in binding capacity to the modified Θ toxins. This result suggested that 1C3 and 2D4 are antibodies recognizing different antigenic sites on Θ toxin. On the other hand, neutralizing antibody 3H10 did not bind to the SRBCM-Θ in spite of good binding to the chemically modified toxins. It is quite possible that 3H10,

Table 2. Binding activity of monoclonal antibodies to θ toxin with different forms

| Antibody | ELISA (U/ml)[a] | | | |
|---|---|---|---|---|
| | θ | SRBCM-θ | DTNB-θ | BIPM-θ |
| 1C3 CF[b] A[d] | 2.7 | 5.0 | 2.9 | 3.2 |
| AF[c] A | 2.7 | 5.0 | 2.9 | 3.2 |
| 2D4 CF A | 2.7 | 4.4 | 5.5 | 5.1 |
| AF A | 2.7 | 5.9 | 9.0 | 6.4 |
| 3H10 CF A | 0.35 | <0.01 | 0.34 | 0.56 |
| AF A | 6.0 | <0.01 | 7.0 | 9.4 |
| Reference | 1.0 | 1.0 | 1.0 | 1.0 |

[a] Antibody titer was measured with microplates coated with native, SRBCM-bound, DTNB-modified, or BIPM-modified θ toxin and expressed as a value relative to the reference antibody.
[b] Antibody from culture supernatant of the hybridoma as described in *Materials and Methods*.
[c] Antibody from ascites fluid as described in *Materials and Methods*.
[d] Antibody purified by protein-A–sepharose.

which shows toxin neutralization, binds to the same site or a site near the one at which the toxin reacts with the cell membrane. To obtain other types of antibodies recognizing antigenic sites on Θ toxin other than the above three antibodies, a second cloning was carried out. Three hybridomas, 1B9, 2C5, and 3F11, were obtained by limiting dilution and were propagated in culture media and in mouse ascites fluid for antibody production. Two antibodies, 1B9 and 3F11, were also type 1 described in Table 1, but it has not been determined whether these two antibodies were distinct from each other or from 1C3 or 2D4. Another antibody 2C5 had binding and neutralizing activities to both Θ toxin and SLO like 3H10, but the neutralization pattern was different from that of 3H10; the end point of neutralization by 3H10 with SLO was very sharp, but that with Θ toxin was gradual. On the other hand, that by 2C5 was gradual not only with Θ toxin, but also with SLO. Therefore, the recognition sites of 2C5 and 3H10 on the Θ toxin and SLO molecules may not be identical. As shown in Table 3A and B, no synergistic effect of these antibodies on binding and neutralizing capacities was observed. The binding activity of these monoclonal antibodies to Θ toxin and SLO was not affected by addition of cholesterol to the microplate coated with each toxin (data not shown).

Next, the kinetics of the antihemolytic effect on Θ toxin and SLO was examined. Both antibodies, 3H10 and 2C5, neutralized both toxins, Θ toxin and SLO, even after binding of the toxins to SRBC at 0°C, although cholesterol could not inhibit hemolysis under such conditions.

Table 3. Binding and neutralizing activities of each monoclonal antibody and their mixtures

| Antibody | ELISA (U/ml)[a] | Neutralizing titer | |
|---|---|---|---|
| | θ | θ | SLO |
| A | | | |
| 1. 1C3 AF[a] A[a] | 2.9 | <10 | <10 |
| 2. 2D4 AF A | 2.9 | <10 | <10 |
| 3. 3H10 AF A | 6.0 | 1,600 | 3,800 |
| 4. 1 + 2[c] | 4.0 | <10 | <10 |
| 5. 1 + 3 | 5.0 | 800 | 1,900 |
| 6. 2 + 3 | 6.5 | 1,600 | 3,800 |
| 7. 1 + 2 + 3 | 5.0 | 1,100 | 1,600 |
| Reference | 1.0 | 250 | 10 |
| B | | | |
| 1. 1B9 AF A | 0.68 | <2 | <2 |
| 2. 3F11 AF A | 0.12 | <2 | <2 |
| 3. 2C5 AF θ[b] | 0.68 | 8 | 4 |
| 4. 3H10 AF θ | 0.40 | 64 | 128 |
| 5. 1 + 2[c] | 0.40 | <2 | <2 |
| 6. 1 + 3 | 0.68 | 4 | <2 |
| 7. 1 + 4 | 0.40 | 32 | 64 |
| 8. 2 + 3 | 0.40 | 8 | 2 |
| 9. 2 + 4 | 0.27 | 32 | 64 |
| 10. 3 + 4 | 0.42 | 16 | 32 |
| 11. 1 + 2 + 3 | 0.49 | 4 | <2 |
| 12. 1 + 2 + 4 | 0.31 | 16 | 32 |
| 13. 1 + 3 + 4 | 0.61 | 32 | 64 |
| 14. 2 + 3 + 4 | 0.31 | 16 | 32 |
| 15. 1 + 2 + 3 + 4 | 0.61 | 16 | 32 |
| Reference | 1.0 | 144 | <10 |

[a] See footnotes *a*, *c*, and *d* of Table 2.
[b] Antibody purified by θ-sepharose, as described in *Materials and Methods*.
[c] Same volume of each antibody was mixed.

Table 4 is a summary of the activities of six monoclonal antibodies that were purified by affinity chromatography with Θ–sepharose and/or protein-A–sepharose from culture supernatant or ascites fluids. As shown in Table 4, nonneutralizing antibodies—1C3, 2D4, 1B9, and 3F11—bound to SRBCM-Θ, but the neutralizing antibodies, 3H10 and 2C5, were not able to bind to such oriented Θ toxin. The passive hemagglutinin (PHA) reaction was also attempted to confirm the binding activity of the antibodies to Θ toxin, by use of formalinized SRBC sensitized with Θ toxin with or without tannic acid. The result of the PHA test confirmed the data concerning binding capacity to SRBCM-Θ obtained by ELISA in Table 4. The four nonneutralizing antibodies agglutinated both sensitized SRBC, but the other two did not.

The hybridomas producing antibodies listed in Table 4 were stable antibody producers; their antibody production ability was not affected by contin-

Table 4. Summary of activities of six monoclonal antibodies[a]

| Antibody | Protein $A_{280}$ nm | ELISA U/ml θ | ELISA U/ml SRBCM-θ | ELISA U/ml SLO | ELISA SLO/θ | Neutralization Titer θ | Neutralization Titer SLO | Neutralization SLO/θ |
|---|---|---|---|---|---|---|---|---|
| 1C3 CF A | 1.37 | 3.39 | 6.64 | <0.001 | | <2 | <2 | |
| AF θ | 0.14 | 0.07 | 0.16 | <0.001 | | <2 | <2 | |
| AF A | 1.06 | 2.49 | 4.63 | <0.001 | | <2 | <2 | |
| 2D4 CF A | 1.21 | 8.90 | 12.99 | <0.001 | | <2 | <2 | |
| AF θ | 0.39 | 2.02 | 2.53 | <0.001 | | <2 | <2 | |
| AF A | 1.45 | 9.50 | 16.15 | <0.001 | | <2 | <2 | |
| 1B9 CF A | 2.20 | 4.44 | 7.55 | <0.001 | | <2 | <2 | |
| AF A | 0.94 | 2.73 | 3.82 | <0.001 | | <2 | <2 | |
| 3F11 CF A | 0.71 | 2.00 | 3.00 | <0.001 | | <2 | <2 | |
| AF A | 1.72 | 8.48 | 14.75 | <0.001 | | <2 | <2 | |
| 3H10 CF A | 0.48 | 0.76 | <0.001 | 11.0 | 14.5 | 80 | 221 | 2.8 |
| AF θ | 0.21 | 0.68 | <0.001 | 16.0 | 23.5 | 76 | 220 | 2.9 |
| AF A | 1.79 | 10.96 | <0.001 | 222.0 | 20.3 | 1,441 | 4,012 | 2.8 |
| 2C5 CF A | 0.90 | 1.31 | <0.001 | 10.7 | 8.2 | 12 | 13 | 1.1 |
| AF θ | 0.68 | 3.88 | <0.001 | 40.3 | 10.4 | 32 | 36 | 1.1 |
| AF A | 0.79 | 4.28 | <0.001 | 45.9 | 10.7 | 33 | 40 | 1.2 |
| Reference | 0.48 | 1.0 | 1.0 | 1.0 | 1.0 | 110 | 5 | 0.045 |

[a] See footnotes of Tables 2 and 3.

uous transfer from mouse to mouse, culture to mouse, or culture to culture.

**Inhibitory activity of the antibodies against cardiotoxicity of Θ toxin and SLO.** Since Θ toxin and SLO showed cardiotoxicity in a heart culture system reported by Honda et al. [9], the inhibitory effect of the six monoclonal antibodies against the toxicity was examined. Heart cells were isolated from 16-day-old mouse fetuses according to the method of Goshima [8]. The antibodies were mixed with Θ toxin (40 ng/10 μl) or SLO (800 ng/10 μl) and were applied to beating cultured heart cells (100 μl) under a phase-contrast microscope. Each toxin stopped the beating of the heart cells and destroyed them within a minute if no antibody was added. Both antibodies, 3H10 and 2C5, with antihemolytic activity neutralized the cardiotoxic activity of Θ toxin and SLO. The other four antibodies with no antihemolytic activity did not have any effect on the cardiotoxicity of the toxins.

## Discussion

Six types of monoclonal antibodies with different combinations of reactivity to Θ toxin and SLO can be considered, as shown in Table 1. In this experiment, only two types, types 1 and 6, of antibodies were obtained. Antibodies 1C3, 2D4, 1B9, and 3F11 of type 1 and 3H10 and 2C5 of type 6 were characterized. Although other types of antibody were not obtained in our experiment, the results reported by Mansa and Kjems [10] suggest the existence of type-4 or type-5 antibodies, which neutralize homologous hemolysin but not heterologous hemolysin. The existence of 3H10 and 2C5, neutralizing both Θ toxin and SLO, however, appeared contrary to the concept that hyperimmunization is necessary to obtain cross-reactive sera to other cytolysins. The fact that the antibodies 3H10 and 2C5 did not react with tetanolysin also showed that antigenic sites recognized by these antibodies were not necessarily common in every thiol-activated cytolysin. Focusing on the characters of 3H10 and 2C5, differences between the reactivity of the two antibodies as shown in Table 4 seem to represent differences in the structure of Θ toxin and SLO. It is well known that the activity of the cytolysins was inhibited by many kinds of substances other than the specific antibody. Therefore, it was necessary to purify the immunoglobulin from the culture or ascites fluid to examine the neutralizing activity of the antibody. Even at the screening step of the hybridoma producing antibody, the results obtained have to be judged very cautiously. In this study, only six monoclonal antibodies were purified and characterized, but when more types of monoclonal antibodies are obtained, the structure–activity relationship among the thiol-activated cytolysins will be clarified.

Through this study, we developed a simple method of purification and estimation of the cytolytic toxins. Theta toxin was purified by affinity chromatography with monoclonal antibody (3H10)–combined sepharose. The toxin was eluted with 3 *M* NaSCN without any decrease in the activity. Activity of the toxins was assayed on microplates coated with SRBC membrane by the ELISA method. This concept and technique are the same as for the method of pertussis toxin titration reported previously [14]. The ELISA system was almost the same as the hemolysis assay with respect to sensitivity, but was superior in reproducibility and quantification.

A speculative model concerning the mechanism of hemolytic action by SLO has been shown; there are two active sites on the toxin molecule, one for fixation to the cell membrane and the other for lysis [1, 11, 13]. It appears that both neutralizing antibodies, 3H10 and 2C5, have an affinity for the lytic site, which does not have affinity for cholesterol. We were not able to obtain the monoclonal antibody directed at the fixation site, but the existence of the neutralizing antibodies that inhibit hemolysis even after binding of the toxins to SRBC seems to support the above speculation [1, 11, 13].

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