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Murine Monoclonal Antibodies Neutralizing the Cytotoxic Activity of Diphtheria Toxin

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SUMMARY In this study, murine monoclonal antibodies that specifically bound to the A and B subunits of diphtheria toxin (DT) were produced by conventional hybridoma technology using the spleens of BALB/c mice immunized with diphtheria DTP vaccine and CRM_{197}. Monoclonal antibodies specific to the A subunit, *i.e.* clone AC5, as well as those specific to the B subunit, *i.e.* clone BB7, could neutralize the DT-mediated cytotoxicity to Vero cells in microcultures. The DT neutralizing mechanisms have yet to be determined. The MAbBB7 is hypothesized to either interfere with the DT receptor binding or with the pore forming function of the T domain of the B subunit. The MAbAC5 could neutralize the DT mediated cytotoxicity when mixed with the DT before adding to the Vero cell culture thus suggesting that the antibody interfered with the translocation of the A subunit. The A subunit-antibody complex might be too large to pass through the membrane channel formed by the T domain and thus prevent the accessibility of the A subunit to the cytosolic target. It is also possible that the MAb AC5 blocked the enzymatic active site of the enzyme catalytic subunit. While further experiments are needed to localize the epitopes of the two MAbs on the holo-DT in order to reveal the DT neutralizing mechanisms, both MAbs in their humanized forms have a high potential as human therapeutic antibodies for diphtheria.

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Diphtheria is a disease caused by the toxin of lysogenic *Corynebacterium diphtheriae* bacilli that superficially colonize the upper respiratory tract of humans. The pathogenicity of *C. diphtheriae* has been extensively reviewed by Pappenheimer and Gill[1] and Collier.[2] Under the iron limited condition in the human throat, the growing bacteria secrete an exotoxin, named diphtheria toxin (DT). The genes encoding the DT production are contributed by a lysogenic bacteriophage; thus, non-lysogenic strains of *C. diphtheriae* are not pathogenic.[3-5] DT is produced as a single polypeptide proenzyme with two disulfide links. The polypeptide must be nicked by the co-secreted protease and reduced to release the N-terminal A, and the C terminal B fragments.[6] The A subunit (193 amino acid residues) is an enzyme catalytic domain and functions in the host cell cytosol by catalyzing the NAD^+-dependent ADP-ribosylation of the host cell elongation factor (EF)-2, rendering the target molecule inactive; thus, the protein synthesis is inhibited, leading to cell death.[7-9]

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The C-terminal B subunit (342 amino acid residues) consists of two functionally different domains, the receptor binding (R), and the translocation (T) domains. For pathogenicity, the holo-DT toxin, *via* the R domain, binds to a receptor of the sensitive eukaryotic cells and the DT-receptor complex is endocytosed principally *via* the clathrin-coated pit.[10,11] A low pH in the late endosome mediates a conformational change of the B subunit as well as the insertion of a hairpin T domain into the endosomal membrane facilitating the A subunit translocation across the membrane into the host cell cytosol.[12,13] The most common clinical features at the prelude of diphtheria are fever, sore throat, malaise and headache which are indistinguishable from a common cold and other febrile illnesses. Diphtheria clinically manifests in two forms, laryngeal and systemic diphtheria.[13] In the laryngeal form, toxigenic *C. diphtheriae* bacilli in the nasopharynx produce DT which attacks larynx and tonsils causing cell death of the local tissue as well as of infiltrating inflammatory cells. Within 2-3 days after the bacterial colonization, a pseudomembrane composed of death cells, necrotic tissue and growing bacilli is formed over the initial site of the infection. This feature is diagnostic of diphtheria. Without prompt and adequate treatment, the diphtheria pseudomembrane will spread and cover the entire larynx causing narrowing of the respiratory tract and difficulty in breathing. The patient may suffocate and die from brain anoxia. Systemic diphtheria is caused by a spread of the locally produced DT through the blood circulation. The pivotal tissues frequently targeted by the DT are the heart muscle and the nerve cells causing myocarditis, disturbance of the heart rhythm and heart failure, and polyneuritis, respectively.[14]

Treatment of diphtheria with antibiotics after the clinical symptoms have manifested and the laryngeal pseudomembrane has formed (diagnosis is often made at this stage) is rather ineffective as by then the DT has already reached and damaged the target organs. The usual treatment of diphtheria is by infusing a ready made immune globulin from either homologous (serum of convalescing or immunized-individuals) or heterologous sources (such as horse immune serum/murine monoclonal antibody) that neutralize the circulating DT. However, the supply of human derived-immune serum is limited and the ethical issue is of particular concern. The polyclonal antibody from horse often cause hypersensitivity (allergy and/or serum sickness) in the recipient. Moreover, a prolonged immunization schedule is required before a satisfactory level of antibody is reached. Murine monoclonal anti-DT antibody have been used as an alternative to the horse anti-serum after the invention of the hybridoma technology by Kohler and Milstein in 1975.[15] However, the preparation still evokes an anti-isotype response, *i.e.* human anti-mouse antibody (HAMA). The murine molecule needs to be humanized by antibody engineering technology in order to abrogate the undesirable immunological response.

This study aimed at producing murine monoclonal antibodies which could readily neutralize DT-mediated-cytotoxicity as a basis for humanized derivatives with the same epitope binding specificities in order to create a better alternative for diphtheria treatment.

MATERIAL AND METHODS

Diphtheria-tetanus-pertussis vaccine (DTP), diphtheria toxin (DT) and non-toxic DT derivative (cross-reacting material 197; CRM_{197})

Alum adsorbed diphtheria, tetanus and pertussis vaccine (DTP) was purchased from Aventis Pasteur, France. Diphtheria toxin (DT) and the non-toxic DT counterpart, *i.e*. cross-reacting material 197 (CRM_{197}) were products of Sigma Chemical Company, USA.

Separation of the A and B subunits of CRM_{197}/DT

To separate the A and B subunits of CRM_{197} or DT, 10 µg of toxin was added into 13 µl of buffer (50 mM Tris-HCl, pH 8.2 and 1 mM EDTA). Then 0.5 µl of trypsin (0.5 g/l) was added into the mixture and incubated at 25˚C for 30 minutes. Dithiothreitol (DTT) was added to a final concentration of 0.1 M and the mixture was further incubated at 37˚C for 90 minutes.

Heterologous antigens used for screening the antigenic specificities of the polyclonal and monoclonal antibodies

Table 1 shows a list of heterologous bacteria and toxins used for screening the antigenic reactivi-

Table 1 Heterologous bacteria and toxins used for cross-reactivity testing of the antibodies in this study

Pasteurella hemolytica	*Streptococcus pneumoniae*
Xanthomonas multophila	*Krebsiella pneumoniae*
Serratia marcescens	*Listeria monocytogenes*
Aeromonas hydrophila	*Enterococcus* spp.
Haemophilus parainfluenzae	*Enterococcus cloaceae*
Moraxella catharalis	*Pseudomonas aeruginosa*
Moraxella lacunata	*Escherichia coli*
Moraxella spp.	*Neisseria meningitidis*
Non-toxigenic *Corynebacterium diphtheriae*	*Neisseria gonorrhoeae*
Toxigenic *Corynebacterium diphtheriae*	*Shigella boydii*
Citrobacter diversus	*Shigella sonnei*
β-hemolysis *Corynebacterium* spp.	*Shigella flexneri*
Staphylococcus aureus	*Vibrio cholerae*
Methicillin resistant *Staphylococcus aureus* (MRSA)	*Vibrio parahaemolyticus*
Non-coagulase *Staphylococcus aureus*	*Vibrio* spp.
Staphylococcus epidermidis	*Proteus sturtii*
Staphylococcus saprophyticus	*P. vulgaris*
β-hemolytic *Streptococcus* spp.	Pertussis toxin
α-hemolytic *Streptococcus* spp.	Tetanus toxin
Group A *Streptococcus* spp.	*Nocardia* spp.
Group B *Streptococcus* spp.	*Edwardsiella tarda*

ties of the antibodies secreted by the polyhybrid cells and hybridomas of this study.

Hybridoma and monoclonal antibody (MAb) production

Hybridomas and MAb were produced as previously described.[16] All animal experiments were performed following the guidelines of the National Research Council of Thailand and were approved by the Ethics Committee of the Faculty of Allied Health Science, Thammasat University, Thailand. Male BALB/c mice, 4-6 weeks old, were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. Each mouse was pre-bled and their non-immune sera (NS) were collected. Individual mice were injected intraperitoneally with 50 µl of the alum adsorbed DTP vaccine. All mice were re-immunized two more times at two-week intervals using the same route and the same immunogen but the dose was increased to 150 µl DPT. Four days before the cell fusion, the immune mouse with the highest serum ELISA antibody titer was given an intravenous injection with 2 µg of the CRM_{197} in 0.2 ml NSS. Four days after the intravenous booster, the immune mouse was bled and the serum was subsequently used as an immune serum (IS). The immune spleen cells and myeloma cells (P3x-63-Ag8.653) were fused at a ratio of 10:1 by using polyethylene glycol (PEG) 3,000-3,700 (Sigma) as a fusogen. Hypoxanthine-azaserine (HA) medium was used in the selection of the hybrid cells. The culture plates were incubated at 37°C in a humidified 5% CO_2 incubator and inspected daily for cell growth or contamination using an inverted microscope. The HA culture medium in the plates was changed every 3 days with fresh medium. The spent culture fluids from wells containing growing hybrid cells were checked for the presence of specific antibodies by an indirect ELISA[17] using DT or CRM_{197} as antigen to coat the ELISA wells. The antigenic specificities of the antibodies from each well were checked by Western blotting against SDS-PAGE-separated-CRM_{197} of which the A and B subunits had been separated as described above. The cells from wells whose spent culture medium contained

antibodies reacting to either A or B subunit were selected and subsequently cloned by the limiting dilution method. The monoclonal antibodies from these hybridomas were re-tested against homologous as well as a panel of heterologous antigens (Table 1) by indirect ELISA. Isotypes and sub-isotypes of the MAbs were determined by using a Mouse typer kit (Bio-Rad, USA) which could determine the mouse immunoglobulin sub-isotypes such as IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgE, kappa (κ) and lambda (λ) by ELISA.

To prepare MAb for the DT neutralization assays, the selected hybridomas secreting MAb specific to either A or B subunits of DT were grown in serum free medium (CD medium, GIBCO, NY, USA). The spent culture medium of each hybridoma clone containing secreted MAb was individually harvested at the late log phase of the cell growth. The protein content of each MAb preparation was determined after the preparation was dialysed against PBS.

Indirect ELISA

An indirect ELISA was used to determine the antibody titers of the sera of the immunized mice and for detecting antibodies in the cell culture fluids in screening for positive hybrids. Indirect ELISA was also used for determining specificity *versus* cross-reactivity of the MAbs as follows. The microtiter plates were coated with appropriate antigens (1 μg of non-toxic DT per ml of carbonate-bicarbonate buffer [pH 9.6]) and the antigen-coated plates were incubated at 37°C overnight. The unbound antigens were washed off with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (PBST). The unoccupied sites on the plates were blocked with 1% bovine serum albumin (BSA) in PBS and incubated at 37°C for 1 hour. The excess BSA was washed off, and 100 μl of antibody preparation (serially diluted individual mouse immune sera, negative control serum, IS (positive control serum), diluted or undiluted cell culture fluid) and fresh culture medium or culture fluid of myeloma cells, which served as a negative control or blank, were added to the appropriate wells. The antigen-antibody reaction was allowed to take place at 37°C for 1 hour. After washing thoroughly with PBST, 100 μl of a 1:1,000 dilution of rabbit anti-mouse Ig-horseradish peroxidase (Dakopatts, Glostrup, Denmark) in PBS containing 0.2% BSA and 0.2% gelatin were added to each well and incubated as described above for 1 hour. The unbound conjugate was removed by washing with PBST and the enzyme substrate was added to all wells (100 μl per well). The reaction was allowed to take place in the dark for 30 minutes and was then stopped by adding 50 μl of 1 N NaOH per well. The optical density of each well was measured at absorbance 492 nm with an ELISA reader (Multiscan *EX*; Labsystems, Helsinki, Finland). The ELISA titer of the antibody preparation was defined as the highest dilution of the antibody giving an optical density of ≥ 0.05. One indirect ELISA unit was defined as the smallest amount of the antibody which gave a positive indirect ELISA reaction.

SDS-PAGE and Western blot analysis (WB)

The SDS-PAGE and WB were performed as previously described.[16-18] DT which had been treated with trypsin and dithiothreitol was separated in a 4% stacking gel and 12% polyacrylamide separating gel using a vertical slab gel apparatus (Bio-Rad). WB was performed by transblotting the SDS-PAGE-separated-DT antigen from the gel to a nitrocellulose membrane (NC). After blotting, empty sites or the membrane blots were blocked with a blocking buffer (3% BSA) for 1 hour. The membranes were then washed to remove the excess blocking substances with a washing buffer (PBS-T) and stripped vertically. Individual antigen blotted NC strips were incubated with antibody preparations (MAb or others) for 1 hour at 25°C on a rocking platform. The membrane was then washed three times with PBS-T. The strips were then put in a solution containing anti mouse-immunoglobulin-horseradish peroxidase conjugate at 25°C for 1 hour with continuous shaking. After washing with the washing buffer, the strips were placed in a freshly prepared substrate solution until the protein band(s) appeared. The membranes were finally thoroughly washed with distilled water (DW) until the background was clear.

Cytotoxic assay and determination of the cytotoxic dose 50 (CD_{50}) of DT

A microcytotoxicity assay was performed using Vero cells *ex vivo* cultures.[19,20] One hundred microliters of two-fold serial dilutions of the DT in

Dulbecco's Modified Eagle's Medium (DMEM) were plated in triplicate wells of 96-well plates and 100 µl of Vero cell suspension at 10^5cells/ml were added to each well. After 48 hours of the cells-toxin incubation at 37°C in a 5% CO_2 incubator, the cells were examined for cytopathic effects (CPE) using an inverted microscope. Individual wells were then washed gently with PBS, pH 7.4, to remove detached cells, cell debris and fluid. The still attached-cells were stained with 0.13% crystal violet for 20 minutes; then the wells were gently rinsed with soft running water until all excess stain was removed and the plates were air-dried. Individual wells were observed under an inverted microscope and the 50% cytotoxic dose (CD_{50}) of DT (amount of DT that caused CPE to ~ 50% of cells in the well) was determined. Alternatively, the CD_{50} was quantified by a successive elution of the stained cells from the wells by using 200 µl of 70% ethanol. The optical densities (OD) of the eluted cells were measured at an absorbance (A) of 595 nm (A_{595nm}). The CD_{50} was calculated by using a dose-response curve with logarithmic transformation of the DT concentrations (Logistic Dose Response Transition equation, Equation 8013). The curve fitting to the data was constructed by the following equation:- $Y=a+/[1+(x/c)^d]$.

The CD_{50} (c) were read at 50% optical density (OD) between maximum OD (b) and minimum OD (a); d is the slope of the curve.

Toxin neutralization test[20]

MAb specific to either A or B subunits of DT were tested against the holo-DT, to determine the DT neutralization capacity of the MAb. Fifty microliters of DT (10 CD_{50}) were incubated with an equal volume of MAb (various amounts) in individual wells of the 96-well tissue culture plate and the plate was incubated in an 5% CO_2 incubator at 37°C for 2 hours. Positive CPE wells containing only DT without MAb and negative CPE wells containing MAb were included in the same tissue culture plate. A Vero cell suspension (100 µl containing 10^4 cells) was added to each well. After 48 hours of incubation, the cells in all wells were examined for CPE by an inverted microscope. The detached cells, cell debris and fluid were washed away with PBS, pH 7.4. The still attached-cells were stained with 0.13% crystal violet for 20 minutes, then gently rinsed with soft running water until all excess stain was removed and air-dried. The stained cells were eluted out by using 50 µl of 70% ethanol. The OD of the cells eluted from each well was determined at A_{595nm}. The MAb inhibition dose 50 (ID_{50}) was the dilution/concentra-tion of the MAb in the well that gave half of the OD at A_{595nm} between the maximum OD (corresponding to the MAb dilution/concentration that completely neutralized the 10 CD50 of DT) and the minimum OD (corresponding to the dilution/concentration of the MAb that had no neutralizing effect on 10 CD_{50} of the DT).

RESULTS

Hybridomas and specific monoclonal antibodies to DT

The immune mouse which was used as immune spleen cell donor had an indirect serum ELISA titer of 1:20,480 on the day of the cell fusion. A total of 1.9×10^8 spleen cells were fused with 1.9×10^7 myeloma cells. The fused cells were distributed into 2,040 wells on 34 culture plates. Of the 2,040 wells, 1,409 wells (69%) produced growing hybrids. The culture fluids of these 1,409 wells were screened by indirect ELISA using CRM_{197} as the antigen and culture fluids of 35 wells were positive. After an expanded growth of polyhybrid cells of these 35 wells in 24 well-tissue culture plates, the antibodies in their cell culture fluids were checked for their antigenic specificities by Western blot analysis using DTT and trypsin treated DT as antigen. It was found that the culture fluids of 5 and 2 polyhybrids reacted to the A and B subunits of DT, respectively. Polyhybrid cells from 3 representative wells with different Western blot patterns were subjected to cloning and a total of 18 monoclones (hybridomas) could be obtained. There were 8 monoclones that produced MAb reacting only to the A-subunit and 10 monoclones that produced antibodies specific to the B-subunit (Table 2). Fig. 1 shows reactivities of MAb of representative clones against SDS-PAGE-separated DT which had been treated with DTT and trypsin. The indirect ELISA titers of the spent culture fluids of all of the hybridomas when the cells had grown to the late log phase of growth ranged from 1:128 to 1:512. All MAbs belonged to the IgG2a/κ immunoglobulin. Table 2 shows a list of the hybridomas that secreted MAb specific to the A

Table 2 Monoclones (hybridomas) generated against DT and the MAb titers which they produced

Antigenic specificity to	Hybridoma	Indirect ELISA titer
A-subunit	AB6	1:256
	AB9	1:256
	*AC5	1:512
	AC8	1:256
	AC9	1:256
	AC10	1:512
	AC11	1:512
	AD5	1:512
B-subunit	BB5	1:160
	*BB7	1:256
	BB10	1:160
	BC4	1:160
	BC5	1:160
	BC10	1:256
	BD3	1:160
	BD6	1:256
	BE1	1:160
	BG7	1:256

*MAb that was used in DT neutralization test.

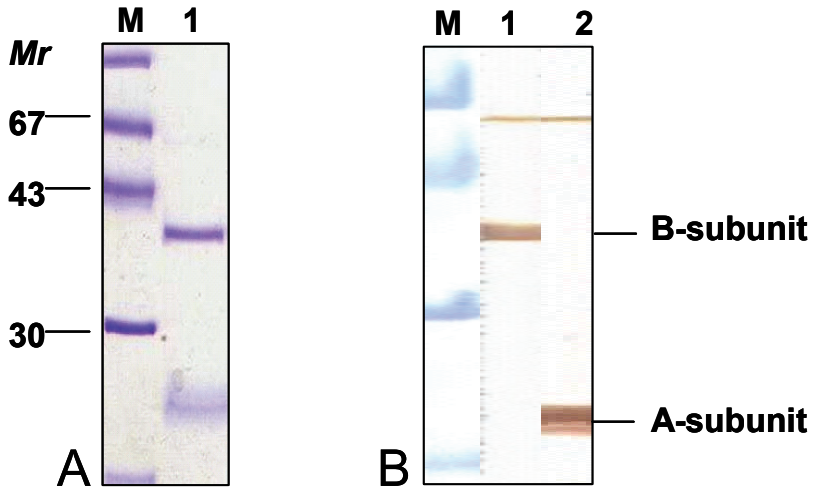

Fig. 1 (A) SDS-PAGE pattern of DT after treatment with trypsin and DTT stained by Coomassie Brilliant Blue R-250 (lane 1); (B), Western blot patterns of MAbs from representative hybridoma clones showing specificity to the A (clone AC5) and B (clone BB7) subunits of DT (lanes 1 and 2, respectively). Lanes M of both (A) and (B), molecular mass standard markers.

or B subunit of DT. The MAb of clones AC5 and BB7 which grew well and produced the highest MAb titers against DT (1:512 and 1:256, respectively) were selected for further use.

Cytotoxic dose 50 (CD_{50}) of DT

Fig. 2 shows Vero cell monolayers in culture and the Vero cytopathic effect (CPE) mediated by

the DT. The amount of DT that killed 50% of Vero cells in tissue cultures (CD_{50}) was 0.0084 ng/well or 0.084/ml (Fig. 3).

MAb mediated-DT neutralization assays

In the DT neutralization assay, the 10 CD_{50} of DT (0.84 ng/ml or 0.084 ng/well) was used in all experiments. Each MAb was two-fold serially diluted from 40 µg/ml to 0.6 ng/ml. The inhibition dose 50 (ID_{50}) of the MAbAC5 and MAbBB7 was 1.0 µg/ml or 50 ng and 3.2 µg/ml or 160 ng, respectively as shown in Fig. 4A and 4B, respectively.

DISCUSSION

Passive immunotherapy of diphtheria using immune serum was pioneered by von Behring, a Nobel Prize laureate, in the 1890s. Subsequently antibody-based therapy, called serum therapy, was practiced for treatment of various infectious diseases. After the discovery of antibiotics in 1930s, however, most infections were treated with chemotherapeutic agents. Nevertheless, antibody still had its niche for many viral infections, envenomation, *e.g.* snake bites, and intoxications, *e.g.* tetanus, pertussis, botulism, as well as diphtheria.[21-23]

For the diphtheria pathogenicity, binding of the R domain of the DT B subunit to the receptor (heparin binding epidermal growth factor-like precursor and CD9) on a human cell is the first important step.[24,25] Thus, blocking and interfering with the function of the R domain is expected to be protective against diphtheria. In fact, the monoclonal antibodies that were the most protective in the cytotoxic assay were shown to inhibit the binding of the toxin to the target cells.[26] After binding to the receptor the holo-DT-receptor complexes are endocytosed into the endosome. At this stage, the acid pH of the endosome mediates a conformational change of the T domain which spontaneously inserts its peptide into the endosomal membrane forming a channel through which the A subunit passes into the cytosol. It can be speculated that the antibody that binds to the T domain may not interfere with the cell entry of the holo-DT but could interfere with the membrane insertion of the T domain, and such block the membrane translocation of the A subunit. Although the epitope specificity of the MAbBB7 that could neutralize the DT mediated cytotoxicity in this study has not yet been identified, it can be speculated that the antibody may inhibit the cytotoxicity by either blocking the binding of the R domain to the receptor or by interfering with the channel formation of the T domain. The MAbAC5 which was specific to the A

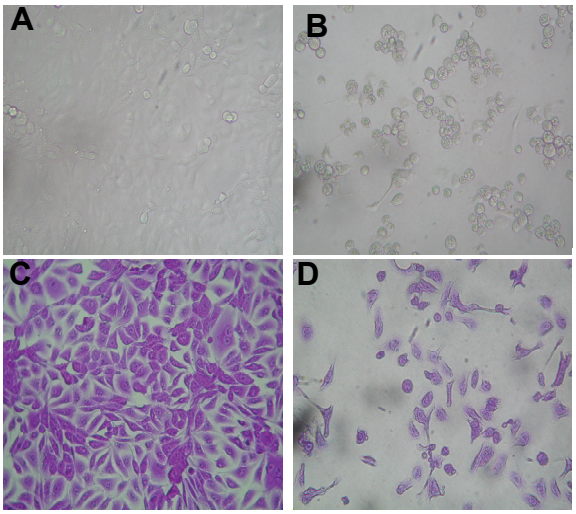

Fig. 2 Cytopathic effect of Vero cells mediated by DT. (A) and (C), Vero cell monolayers unstained and stained with 0.13% crystal violet, respectively; (B) and (D), cytopathic effect (CPE) of Vero cell monolayers mediated by DT unstained and stained with 0.13% crystal violet, respectively.

subunit of DT was found to be more efficient than the MAbBB7 on a weight basis in neutralizing the DT mediated-Vero cytotoxicity. The finding that the intact MAb molecule to the DT A subunit could neutralize the DT activity when mixed with the DT before adding to the Vero cell culture led us to speculate that binding of the antibody to the A subunit before endocytosis of the DT-receptor complex could retain the A subunit in the endosome by forming an A subunit-antibody complex that may be too large to pass through the channel (pore size 18-24 Å in diameter) made by the T domain.[12,13,27] This would prevent accessibility of the A subunit to the cytosolic target. In this instance, the antibody does not have to bind to the enzymatic active site of the A subunit. Alternatively, the antibody might specifically bind at the enzymatic active site of the A subunit and interfere with the catalytic function. Therapeutic antibodies for diphtheria, therefore, can be specific to the R, T and/or A subunit. While further

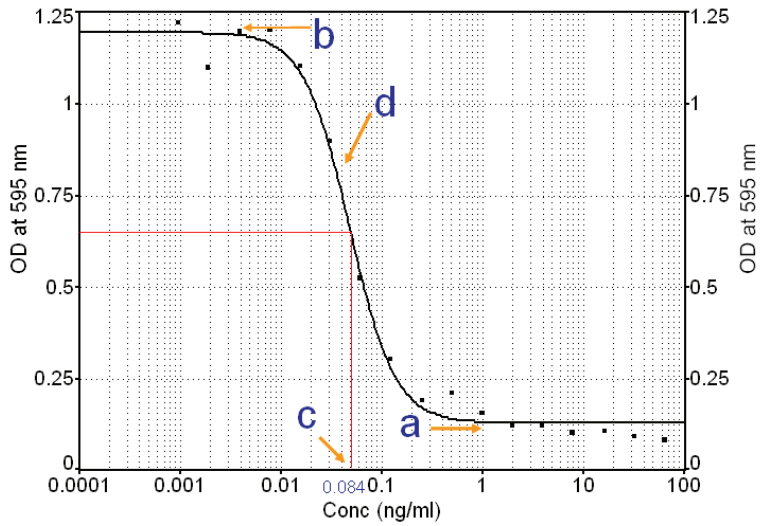

Fig. 3 Dose response curve of Vero cells to DT in the culture. The CD_{50} of the DT was 0.084 ng/ml or 0.0084 ng/well.

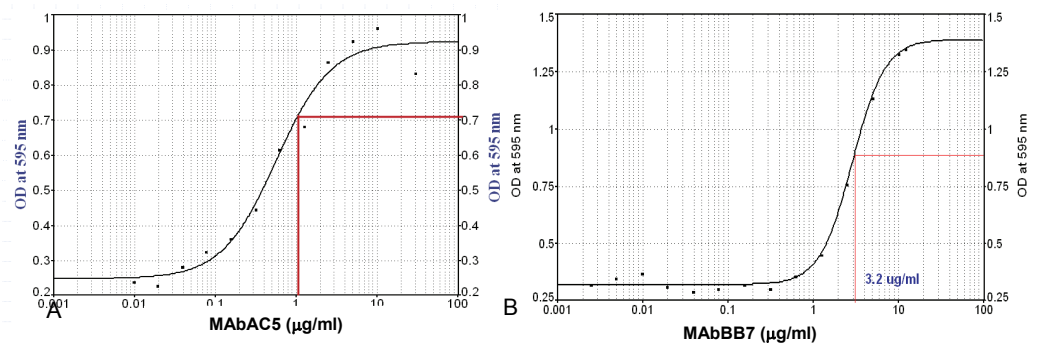

Fig. 4 Results of the neutralization test of the DT mediated-Vero cell cytotoxicity by MAb to the A and B subunits of DT. (A), ID_{50} of the MAbAC5 was 1 µg/ml or 50 ng/well; (B), ID_{50} of the MAbBB7 was 3.2 µg/ml or 160 ng/well.

experiments are needed to localize the epitopes of the MAbBB7 and MAbAC5 on the holo-DT in order to reveal their DT neutralizing mechanisms, both of the MAbs in their humanized forms have a high potential as human therapeutic antibodies for diphtheria.

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