Monoclonal Antibody that Neutralizes Pertussis Toxin Activities

Anek Pootong¹, Prayute Budhirakkul², Pongsri Tongtawe¹, Pramuan Tapchaisri¹, Manas Chongsa-nguan² and Wanpen Chaicumpa¹

SUMMARY  Pertussis or whooping cough is a disease with high mortality among infants and small children. The disease is caused by a gram-negative bacterium, Bordetella pertussis. The superficial colonized bacteria produce a myriad of toxins which enter the circulation causing various pathophysiological changes in the host. Although antimicrobial therapy reduces the number of the coughed out bacteria and also the infectious time of the infected host, but it is not effective in amelioration of the clinical manifestations as the pertussis morbidity is due principally to the pertussis toxin (PT). Antibody-based-therapy is frequently practiced in conjunction with other supportive measure to resuscitate the patient. Nevertheless, human derived antisera against PT is of the limited supply and the ethical concern. Thus in this study a hybridoma clone, i.e. clone PT6-2G6, secreting monoclonal antibody (MAb) specific to the S1 subunit, the active enzyme of the PT that intracellularly ADP-ribosylates the host Gi-protein, was produced. The MAbPT6-2G6 inhibited the in vitro hemagglutination of chicken erythrocytes which is the activity of the B oligomer of PT; thus we hypothesize that the MAb bound to its epitope on the S1 subunit and stereologically hinders the binding sites of the B subunits. The MAb also inhibited ex vivo Chinese hamster ovarian cell clustering and neutralized the in vivo leucocytosis- promotion in mice which are usually mediated by intracellular S1 subunit. The large molecular nature of the intact MAb and its molecular hydrophilicity led us to speculate that the observed PT neutralizing activities of the MAb were due to interfering with the cellular entry of the S1 rather than the intracellular enzyme neutralizing activity per se. While further experiments are needed to pinpoint the MAb neutralizing activity and to identify the amino acid sequence and location of the MAbPT6-2G6 epitope, our findings indicate that this murine MAb, in its humanized-version, should have high therapeutic potential for pertussis.
days after the superficial attachment of the *B. pertussis* at the respiratory epithelium and is characterized by common cold-like symptoms with mild intermittent cough and low grade fever. The cough is later intensified, becomes paroxysmal and spasmodic which are the characteristics of the second phase. During the paroxysmal phase thick mucus plugs may block the respiratory tract and infants may have sustained apnea, hypoxia and collapse during the coughing spells. Pneumonia is not uncommon while neurological complications, ranging from tremor and seizure to severe neurological problems, are frequently observed. Among the *B. pertussis* virulence factors, pertussis toxin (PT), a 105 kDa hexamer, is the principal toxic component. PT is an A-B₅ toxin consisting of A or S₁ subunit which is an ADP-ribosyltransferase and a B pentamer made up of two dimers, *i.e.*, S₂S₄ and S₃S₄, and one S₅, which is responsible for binding of the holotoxin to the host cell surface. PT exists in two forms, cell-bound and secreted. The cell-bound PT functions as one of the bacterial colonizing factors.⁵,⁶ Once the PT bound to the target host cell via its B subunits, the A (S₁) subunit is cleaved-off and traverses the plasma membrane into the cytoplasm where it transfers ADP-ribose moiety from NAD to a cysteine residue at position 352 of the α-subunit of the membrane-bound inhibitory guanosine-binding protein (Gi-protein). The normal function of this Gi protein [adenylyl cyclase inhibition by means of guanosine triphosphate (GTP) hydrolysis to guanosine diphosphate (GDP)], is disrupted. The result is a sustained adenylate cyclase activation and, in the effect, the massive increment of intracellular cyclic adenosine mono-phosphate (cAMP).⁷ The increase of intracellular cAMP causes cellular function disruption including defect in all steps of phagocytic activity and/or cytotoxicity of the host cells.⁸ PT via the S₁ subunit causes also systemic leucocytosis, termed leucocytosis-promotion (LP), histamine sensitization and islet cell activation with massive release of insulin.⁹ The S₁ subunit of PT was found to mediate characteristic rounding and clustering of the Chinese hamster ovarian (CHO) cells *ex vivo*.¹⁰ The PT-B subunit not only binds to the target cell for S₁ intracellular delivery but was found also to have immuno-(pathological) potentiating effects, *e.g.*, T-lymphocyte mitogenicity.¹¹ The B pentamer *via* its S₂S₄ and S₃S₄ dimers causes chicken erythrocyte agglutination *in vitro*.¹⁰

Treatment of *B. pertussis* infection by using the antimicrobial agents may reduce the number of bacteria coughed out by the patient and the patient’s infectious time, which is important in reducing the bacterial spread. However, the drugs are not effective in amelioration of the pertussis during the catarhal and paroxysmal stages as the clinical manifestations and the pathophysiology in the patients are caused by the circulating toxins readily secreted by the *B. pertussis* confined in the respiratory tract. Frequently, human immune globulin is used in conjunction with supportive measures such as artificial respiration for treatment of pertussis. The therapeutic antibodies are, however, derived from plasma pool of donors immunized with pertussis toxoid which often raises practical problem (inadequate supply) and ethical issue. Murine monoclonal antibodies specific to the PT in their humanized-form would serve as an alternative of the human therapeutic antibody. Thus, in this study we produced murine hybridomas secreting monoclonal antibodies (MAb) that neutralize activities of PT.

**MATERIALS AND METHODS**

**PT, pertussis toxoid and other antigens**

Purified pertussis toxoid (PT) was a gift from BIKEN Institute, The Research Foundation of Microbial Disease of Osaka University, Japan. Pertussis toxoid was from the Division of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand. Tetanus and diphtheria toxins were from the Government Pharmaceutical Organization (GPO), Thailand. Cholera toxin and streptolysin O were purchased from Sigma Chemical Co., USA. Whole cell homogenates of microorganisms commonly found in the human respiratory tract were prepared as previously described.¹² List the antigens used in this study are shown in Table 1. Murine MAb specific to the A subunit of cholera toxin produced in our laboratory was used as a negative MAb control.

**Hybridomas and monoclonal antibody (MAb) production**

Hybridomas secreting specific monoclonal antibodies to PT protein were produced as previously described.¹³ Female BALB/c mice (8 weeks old)
MONOCLONAL ANTIBODY TO PERTUSSIS TOXIN

### Table 1
Reactivity of monoclonal antibodies secreted by the established hybridomas to homologous and heterologous antigens when tested by the indirect ELISA

<table>
<thead>
<tr>
<th>Antigenic type</th>
<th>Toxin/organism</th>
<th>PT1-2E6</th>
<th>PT1-3B10</th>
<th>PT5-2C11</th>
<th>PT6-2H7 *</th>
<th>PT6-2G6</th>
<th>PT7-1H11</th>
<th>PT7-2F6</th>
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<td><strong>Toxins</strong></td>
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<td></td>
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<tr>
<td>Pertussis toxin</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
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<tr>
<td>Tetanus toxin</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Diphtheria toxin</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Cholera toxin</td>
<td>+</td>
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<td>Streptolysin O</td>
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<td>Acinetobacter spp.</td>
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<td>Bordetella pertussis</td>
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<td>Haemophilus influenzae</td>
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<tr>
<td>Klebsiella pneumoniae</td>
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<td>Staphylococcus coagulase negative</td>
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<tr>
<td>Streptococcus pneumoniae</td>
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<td><strong>Yeast</strong></td>
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<tr>
<td>Candida albicans</td>
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* MAb secreted by this clone was further used in the subsequent experiments

were kindly provided by the Armed Forces Research Institute of Medical Sciences (AFRIMS), U.S. Component, Bangkok, Thailand. The mice were pre-bled before being injected intraperitoneally with 1 μg of PT in 200 μl NSS emulsified in equal volume of Freund’s complete adjuvant. The mice were given two booster doses at two week-intervals with the same dose of the antigen in Freund’s incomplete adjuvant. Fourteen days after the last booster, all mice were bled and their sera were assessed for antibody titers against the PT by an indirect ELISA. An immune mouse showing the highest serum titer was used as a spleen cell donor in the hybridoma production. The selected mouse was given an intravenous injection with 15 μg of pertussis toxoid in 200 μl NSS. Three days later, the mouse was bled to collect the immune serum (IS) then it was sacrificed. Spleen cells of this mouse were fused with P3X-63-Ag8.653 myeloma cells by using polyethylene glycol 4,000 as a fusogen at the ratio of ~10 spleen cells to one myeloma cell. Spent culture medium in wells containing growing hybrid cells were tested for antibodies against the homologous antigen by indirect ELISA. Cells from the antibody-positive wells were subjected to monocloning by limiting dilution method using spleen cells of a naïve BALB/c mouse as feeder cells. Culture fluids of individual hybridoma clones were retested against homologous and heterologous antigens by the indirect ELISA. Clones secreting monoclonal antibodies (MAb) reactive only to PT were selected. Antigenic specificities of the MAb secreted by individual clones were determined by Western blot analysis (WB) against the SDS-PAGE separated-PT. Immunoglobulin types and subtype(s) of the MAb were identified using Mouse Typer Sub-isotyping kit (Bio-Rad, CA, USA).

A hybridoma secreting MAb specific to PT was grown in serum free medium to late log phase.
The cell spent medium was collected, concentrated by pressure filtration method (Amicon, MA, USA) and purified by using a protein G affinity column. Protein concentration of the IgG preparation was determined.

Indirect ELISA

An indirect ELISA was used for determining antibody titers of sera from immunized mice and for detecting antibodies in the cell spent culture media for screening of positive hybrids. The technique was also used for determining specificity versus cross-reactivity of the MAbs. Wells in polystyrene microtiter plates (Costar, Corning, NY, USA) were individually coated (100 μl per well) with appropriate antigens (1 μg of bacterial toxin per ml or 50 μg of bacterial homogenate per ml of carbonate-bicarbonate buffer, pH 9.6). The plates were incubated at 37°C in a humid chamber for 1 hour then all wells were washed three times with phosphate buffered saline (PBS) containing 0.5 % Tween-20 (PBS-T) to remove the unbound antigens. The remaining unoccupied sites in each well were blocked by incubating with 200 μl of a blocking buffer [1% bovine serum albumin (BSA) in PBS] at 37°C for 1 hour. The excess BSA was washed off, and 100 μl of antibody preparation was added to the well. The antigen-antibody reaction was allowed to take place at 37°C for 1 hour. After washing with PBS-T, 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 was added to each well and incubated as above. The unbound conjugate was removed by washing with PBS-T. The enzyme substrate was added to all wells (100 μl per well). The reaction was allowed to take place in the dark for 10 minutes and was then stopped by adding 50 μl of 1 N NaOH per well. The optical density of the content in each well was determined at 492 nm using an ELISA reader (Titertek Multiskan, Flow Laboratory, Switzerland). The ELISA titer of an antibody preparation was the highest dilution of the preparation giving an optical density at or higher than 0.05 above the blank (content of well that PBS was added instead of the antibody). One indirect ELISA unit was defined as the least amount of the antibody preparation which gave a positive indirect ELISA reaction.

SDS-PAGE and Western blot analysis (WB)

SDS-PAGE was carried out in a vertical slab gel apparatus (Mini-PROTEAN-III, Bio-Rad, USA) according to the previously described method. PT in the sample buffer was heated at 56°C for 30 minutes before loading onto a 4% stacking gel and 15% acrylamide separating gel were used in the procedure. WB was performed by electro-transblotting the SDS-PAGE separated PT from the gel to a nitrocellulose (NC) membrane. The unoccupied sites on the NC membrane were blocked by placing the membrane in a solution of a blocking buffer (3% BSA, 5% gelatin in PBS) at 25 °C with gentle rocking for 1 hour. The membrane was washed with PBS-T and incubated in an MAb solution for 1 hour, washed and placed in a solution of rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dakopatts) at a dilution of 1:1,000 in PBS containing 1% BSA and 1% gelatin at 25°C for 30 minutes. After excessive washing with the washing buffer, the membrane was placed in a freshly prepared substrate solution for 5 minutes, washed with distilled water, and air dried.

Hemagglutination assay (HA) and hemagglutination-inhibition (HI) test

Hemagglutination assay (HA) was performed as previously described. Briefly, 50 μl of 0.7% chicken erythrocytes in PBS (v/v) was mixed with serially diluted PT. The preparation was incubated at 25°C for 30 minutes. The least amount of PT that cause complete agglutination of the red blood cells (hemagglutination dose 100; HD100) was determined by observing the hemagglutination by light microscopy at 100x magnification. For the HI test, one HD100 of PT in 25 μl of PBS was incubated with various amounts of MAb at 25°C for 30 minutes. Fifty μl of 0.7% chicken erythrocyte suspension was added to each PT-MAb mixture and the preparation was kept at 25°C for 30 minutes. The lowest amount of the MAb that could inhibit the hemagglutination caused by the HD100 of PT was taken as an HI dose.

Chinese hamster ovarian (CHO) cell assay

CHO cells cultured in the presence of PT display a characteristic clustering pattern. In this...
study, the MAb specific to S1 was tested for its ability to inhibit the CHO cell clustering (CC). CHO cells (strain K1) were cultured in a 25 ml-tissue culture flask (Corning) in a 10% fetal bovine serum supplemented-RPMI-1640 medium containing 1% antibiotics, i.e. 100 units penicillin and 100 μg streptomycin per ml (complete RPMI medium) in a 5% CO2 incubator at 37°C until their confluent growth was obtained which took about 2-4 days. The cells were trypsinized, harvested from the flask and adjusted to 2 x 10⁵ cells/ml with the serum supplemented-RPMI medium. Wells in the 96-well tissue culture plate (Costar) were added with 100 μl of the medium containing various amounts of PT. CHO cells (100 μl) were added to individual PT containing wells (test wells) and also to the wells containing only the RPMI medium (negative control wells). The plate was incubated in the 5% CO2 incubator at 37°C for 16-24 hours. The least amount of PT that caused clustering of ~50% of the cells in the well as compared to the negative control well was taken as one cell clustering dose 50 (CCD50). For the CHO cell clustering inhibition (CCI) assay, various amounts of the MAb in 50 μl of complete medium was added to duplicate wells in a tissue culture plate and one CCD50 of the PT was added to each well. Duplicate wells containing only the PT (positive control wells) were included in the plate. After incubation at 25°C for 30 minutes, CHO cells in 100 μl of the RPMI medium were added to each culture well and the plate was incubated in the 5% CO2 incubator as above. The cells in all wells were inspected every 6 hours under 100x magnification light microscopy. The minimum amount of the MAb that could rescue the cells from clustering when 50% of cells in the positive control wells showed the characteristic clustering was determined.

**Leukocytosis-promotion (LP) inhibition test**

On day 0, EDTA blood samples were taken individually from 30 female, 6 week old BALC/c mice. The number of white blood cells (WBC) in each sample was counted as the baseline data. The mice were then divided into 5 groups (groups 1-5). Mice of groups 1 and 2 were each injected intravenously with 100 μl PBS alone (negative LP control) or PBS containing 500 μg of MAb specific to the A subunit of cholera toxin plus 100 ng PT (positive LP control), respectively. Mice of groups 3-5 were intravenously injected with the same amount of PT previously incubated with three different concentrations of S1 specific MAb. Four days post-injection (day 3), all mice were bled and their WBC were counted. The WBC count of each mouse on day 3 was subtracted with baseline WBC count of day 0 of that particular mouse to obtain the ∆WBC. The ∆WBC of mice of groups 3-5 were compared with the ∆WBC of group 1 (negative LP control) and group 2 (positive LP control) by using Mann Whitney U test. Difference at \( p < 0.05 \) was statistically significant.

**RESULTS**

**Hybridomas and specific MAb to PT**

The immunized mouse which was used as the immune splenocyte donor had serum ELISA titer of 1:64,000 against PT. A total of 7.8 x 10⁷ spleen cells were obtained from this mouse and were fused with about 7.8 x 10⁷ myeloma cells. Culture fluids from a total of 370 wells which revealed growing cells were tested for antibodies against the homologous antigens by the indirect ELISA, and the spent culture fluids from 45 wells (12.16%) were positive. Cells of only seven wells were found to retain the antibody producing capability after sub-culturing. The spent culture fluids of cells from these wells (PT1 to PT7) were subjected to WB analysis against the SDS-PAGE separated-PT. It was found that antibodies from cells of PT1 well gave faint bands to S1, S2 and S3; antibodies from PT2 and PT3 wells gave weak Western blot result; antibodies from PT4 well gave weak reaction against S3 subunit; and antibodies from PT5, PT6 and PT7 wells showed intense bands against S1 subunit (data not shown).
Fig. 1 Western blot analysis (WB) pattern of monoclonal antibodies in spent culture fluids of the seven monoclonal antibodies against the SDS-PAGE-separated PT. Lane 1, mouse immune serum; lanes 2-8, MAbPT1-2E6, MAbPT1-3B10, MAbPT5-2C11, MAbPT6-2G6, MAbPT6-2H7, MAbPT7-2F6 and MAbPT7-1H11, respectively. Numbers at the left are relative molecular masses (Mr).

**Inhibition of PT mediated- hemagglutination by MAbPT6-2G6**

Because the cells of the clone PT6-2G6 were healthy, grew fast and secreted high titer of MAb (MAbPT6-2G6) against S1 subunit of PT, this clone was selected for further use.

In the hemagglutination assay (HA), the minimum amount of PT that caused complete hemagglutination of the chicken erythrocytes (HD100) was found to be 125 ng (Fig. 2, A). The hemagglutination mediated by this amount of PT was readily inhibited by 6.25 mg of MAb PT6-2G6 (Fig. 2, B).

**Neutralizing activity of MAbPT6-2G6 on PT mediated-CHO cell clustering**

The minimum amount of PT that could cause 50% characteristic CHO cell clustering (CCD50) was 5.6 pg (Fig. 3). The MAb PT6-2G6 at 32.5 μg could mediate ~50% inhibition of CC caused by a CCD50 of PT.

**Neutralization of PT mediated-leucocytosis-promotion by the MAbPT6-2G6**

Fig. 4 shows neutralizing activity of MAbPT6-2G6 against leucocytosis-promotion effect mediated by PT in mice. Mice of group 1 were injected individually with PBS (negative LP controls), mice of group 2 received PT previously incubated with monoclonal antibody to cholera toxin A subunit (positive LP controls), and mice of groups 3-5 were injected with PT incubated with various amounts of MAbPT6-2G6. It was found that the MAbPT6-2G6 could cause statistically significant decrease of LP mediated by the 100 ng of PT in a dose dependent manner (MAb 500 μg > 250 μg > 125 μg). However, the decrease of LP mediated by the MAb did not abrogated the PT mediated LP completely as the △WBC of groups 3-5 were still significantly higher than that of group 1 (negative LP control).

**DISCUSSION**

Ability of specific antibody to protect against disease, *i.e.* diphtheria, was first discovered by von Behring and Kitasato in the early 1890s. Since then the antibody based-therapy, called serum therapy, was practiced for treatment of various infectious diseases in human. The discovery of sulfonamides and antibiotics in the mid 1930s, however, made a change in the treatment strategy of infectious diseases and the serum therapy was mostly replaced by the antimicrobial chemotherapy. Nevertheless, the antibody still retains its therapeutic niche for many viral infections as well as for envenomation, *e.g.* snakebite, and bacterial intoxication, *e.g.* tetanus, botulinum and pertussis.17,18 For pertussis, even though the effector mechanisms of protective immunity against the disease is not completely understood and may involve both humoral and cell mediated immune mechanisms,19 evidences in the literature have pointed out that specific antibodies to PT play important role in amelioration of the pertussis severe morbidity and in protection against the *B. pertussis* infection.20,21

In this study, murine hybridomas, *i.e.* clones PT5-2C11 and PT6-2G6 secreting monoclonal anti-
Fig. 2  Hemagglutination assay for determining HA100 of PT (A) and hemagglutination inhibition assay for determining the HI activity of MAbPT6-2G6 (B). The HA100 of PT was 125 ng (arrow in A) and the HI100 of the MAbPT6-2G6 was 6.25 μg (arrow head in B). +, positive HI; -, negative HI; ±, partial HI reaction.

Fig. 3  Chinese hamster ovarian (CHO) cell clustering phenotype. Left, Monolayer of normal CHO cells in tissue culture; Right, characteristic clustering (CC) of CHO cells mediated by PT.

bodies reacted specifically to S1 subunit of PT were produced. The MAbPT6-2G6 was tested for the neutralizing activity against PT mediated-functions in vitro, ex vivo and in vivo. Our finding that intact MAbPT6-2G6 could readily inhibit the in vitro effect of the PT mediated chicken erythrocyte agglutination, which is the normal activity of the S2S4 and S3S4 dimers of the B oligomer of PT, implies that the epitope of this MAb on the S1 subunit may be in the juxtaposition with the hemagglutination site(s) of the B subunits; thus binding of the MAb to the epitope would stereologically hinder the hemagglutination sites of the B pentamer.
Although the molecular events involved in the cellular entry of PT is not well understood, evidence suggested that after binding of the B oligomer to the host cell membrane, the S1 subunit may traverse across the plasma membrane instead of using the receptor mediated endocytotic pathway. It is also known that intact antibody molecule, because of its large size and molecular hydrophilicity, cannot normally penetrate the hydrophobic plasma membrane of the living cells and thus is inaccessible to its intracellular target. The findings that previous incubation of the PT with the MAbPT6-2G6 which is specific to S1 subunit could inhibit the CHO cell clustering and leucocyte-promotion activities are interesting because these PT functions are mediated by the ADP-ribosyltransferase activity of the intracellular S1. Neutralization of the PT by the intact MAb in this study should not be mediated by interfering the enzymatic function of the S1 by MAb per se, but rather preventing the intracellular entry of the S1 by forming PT-antibody complexes and/or stereologically interfering with binding of the B oligomer to the host cell membrane. We, therefore, speculated further that the epitope of the MAbPT6-2G6 should be exposed on the surface of the PT holotoxin in order to be accessibly bound by the MAb and near to the membrane binding site(s) of the B pentamer. While further experiments are needed to identify the amino acid sequence and location of the MAbPT6-2G6 epitope, our findings demonstrate that this murine MAb, in its humanized-version, should have high therapeutic potential for pertussis.

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