Arch. Pharm. Res. **15**(2), 146-151 (1992)

Effects of Pertussis Toxin on Macrophage Activation

Suck Ihn Lim and Nyeon Hyoung An*
College of Oriental Medicine, Taejeon University, Taejeon 300-716, and
**College of Pharmacy, Wonkwang University, Iri 570-749, Korea*
(Received May 15, 1992)

Abstract □ The aim of this study was to evaluate capability of pertussis toxin(PT) to activate mouse macrophages. The investigations were undertaken to determine whether the role played by this toxin required the A-protomer of the toxin to ADP-ribosylate a guanine nucleotide binding protein (a Class I activity) or was dependent on the binding of B-oligomer of the toxin to the surface of target cells (a Class II activity). The results of these experiments have established that the mechanism of macrophage activation with PT seems to be dependent upon a Class II activity of the toxin.

Keywords □ NAD glycohydrolase (EC 3.2.2.5), pertussis toxin, murine macrophage, guanine nucleotide binding protein.

NAD glycohydrolase (NADase, EC 3.2.2.5) is an enzyme capable of catalyzing the hydrolysis of NAD to form nicotinamide and adenosine diphosphoribose. NADase are widely distributed in nature, being found in microorganisms, plants, and all animal tissues[1-6]. In mamals, NADases are generally found in association with the plasma membranes of most cell types[4,5]. Eucaryocytes possess exclusively NADase activity on the outer plasma membrane surface, which can be solubilized by the treatment of bacterial phosphatidyl inositol specific phospholipase C(PIPLC)[7]. NADase of murine macrophages have been known as a plasma membrane ecto-enzyme and the activities of ecto-NADase were shown to decrease upon the activation of macrophages[8,9].

Pertussis toxin(PT), an exotoxin produced by *Bordetella pertussis*, comprises two components, an enzymatically active A-subunit and a B-oligomer which is responsible for binding of the toxin to eucaryotic cells[10]. The B-oligomer composed of five subunits exhibits certain activities, such as the agglutination of erythrocytes and the mitosis of lymphocytes. The A-subunit catalyzes the ADP-ribosylation of the alpha subunit of GTP-binding proteins (G-proteins) found in eucaryotic cells, leading to elevation of the cAMP formation[11].

Here, the hypothesis which PT may activate the macrophage could be confirmed by demonstrating that the activity of ecto-NADase of macrophages was decreased after treatment of PT *in vivo* and *in vitro*. And also the B-oligomer portion of PT is shown to be responsible for the activation of murine macrophages.

MATERIALS AND METHODS

Materials

Purified pertussis toxin was purchased from List Biological Laboratories (Campbell, U.S.A.), nicotinamideadeninedinucleotide, guanosine triphosphate, adenosine triphosphate, and thymidine from Sigma Chemical Co. (St. Louis, U.S.A.), borane-pyridine complex form Aldrich Chemical Co., Inc. (Milwaukee, U.S.A.), formaldehyde (37℃) from J.T. Chemical Co. (Phillipsburg, U.S.A.).

Methylation of PT

Methylated PT was prepared as described by Ui *et al.*[12]. Briefly, 250 μl of a stock solution of PT (100 μg/ml) in 0.01 M phosphate buffer (pH 7.0)/0.05 M NaCl was mixed with an equal volume of 20 mM formaldehyde and 30 mM borane-pyridine complex (pH 7.0) in an atmosphere of nitrogen at room temperature followed by exhaustive dialysis

against phosphate-buffered saline. As a control, PT was treated in an identical manner with the omission of the incubation step in the presence of formaldehyde and borane-pyridine complex.

Isolation of peritoneal macrophages

Resident macrophages were obtained by lavaging the peritoneal cavity of mice with 5.0 m*l* of cold heparinized (2 U/m*l*) MEM (Flow Laboratories, Inc., McLean, U.S.A.). Cells in the peritoneal fluid were washed and resuspended heparin free MEM containing 10% FCS (Hyclone Sterile System, Inc., Logan, U.S.A.) and incubated in a glass Petri dish at 37℃ within a humidified incubator (5% CO_2) for 90 min. The nonadherent cells were discarded and the macrophages monolayer was collected by treatment with lidocaine.

Effects of PT and mPT on epinephrine-induced hyperglycemia

The ability of PT and mPT to inhibit epinephrine-induced hyperglycemia was evaluated using a slight modification of the method described by Yajima *et al.*[13]. Briefly, The mice were given an I.V. injection of either PTx or mPTx (130 ng). Control animals were given an injection of normal saline. The mice (fasted for 6 hr) were subcutaneously injected with epinephrine (200 μg/kg of body weight) 72 hr later. The blood glucose concentration of each animal was determined immediately before and 60 min after the epinephrine injection. Blood specimens used for blood glucose determination were obtained *via* the retroorbital sinus. The glucose concentration of each sample was determined by the glucose oxidase method[14].

Lymphocytosis-promoting activity of PT and mPT

The lymphocytosis-promoting capacity of PT and mPT was assessed by quantitating the number of lymphocytes in the peripheral circulation of mice that had received an I.V. injection (250 ng) of either PTx or mPTx 72 hr previously. Control animals received in I.V. infusion of normal saline. 50 μ*l* of blood were obtained from each experimental control mice in heparinized capillary tubes. Peripheral leukocyte counts were determined with a hemocytometer and differential analysis of leukocytes was performed on Wrights stained blood smears. The mean values obtained from three similarly treated mice were used as an index of lymphocytosis.

ADP-ribosylation of Gi by PT and mPT

The capacity of PT and mPT to ADP-ribosylate membrane proteins was evaluated using a slight modification of the procedure as described by Backlund *et al.*[15]. Briefly, murine peritoneal macrophages (5×10^6cells/m*l*) were suspended in MEM containing 10% FCS and either PT and mPT (20 ng/m*l*) for 2 hr at 37℃. Crude membrane homogenates were then prepared from both the control and treated macrophages and the membrane preparations were incubated with [^{3}H] NAD and fresh PT. The standard reaction was carried out in 0.1 M potassium phosphate (pH 7.0), containing 10 mM thymidine, 1 mM APT, 0.1 mM GTP, 1.8 μM [^{3}H] NAD (spact : 27.1 Ci/mmol), activated PTx (500 ng) and cell membranes for 1 hr at 37℃ in a total volume of 50 μ*l*. PT was activated by incubation of the toxin for 30 min at room temperature in 50 mM potassium phosphate buffer, pH 7.0 containing 25 mM dithiothreitol. Membranes were washed free of unreacted NAD and the amounts of incorporated radioactivity were counted with a scintillation counter (Packard Instrument Co., Inc., Dower Grove, U. S.A.). For autoradiogram [^{3}H] NAD was replaced by 6 μm [^{32}P] NAD. After membrane pellet was precipitated by 20% trichloric acid, ADP-ribosylated membrane proteins were identified by autoradiography following electrophoresis by 12% SDS-polyacrylamide gel.

Assay of NADase activity

The method was a modification of the assay described by Kaplan[1]. The reaction mixtures contained 1 mM β-NAD, 50 mM potassium phosphate buffer, pH 7.5, and NADase, to a final volume of 0.5 m*l*. After incubation at 37℃ for 10 min, 3 m*l* of 1 M potassium cyanide was added, and the mixture was read at 325 nm.

RESULTS

PT and methylated PT can ribosylate G protein of macrophages

The biological activities of PT on the cells have been divided into two general categories by the actions of A-protomer and B-oligomer of PT, which have been termed Class I and Class II activities.

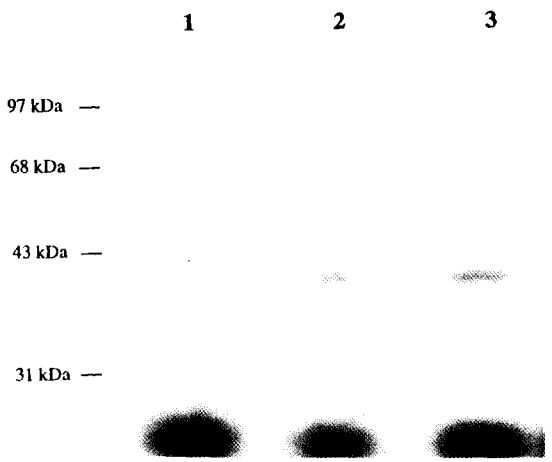

Fig. 1. Effects of PT and mPT on the [^{32}P] ADP-ribosylation of macrophage membranes.
Lane 1, minus toxin; lane 2, 10 mγg/m*l* PT; lane 3, 10 μg/m*l* mPT. Molecular weight markers were indicated as kDa.

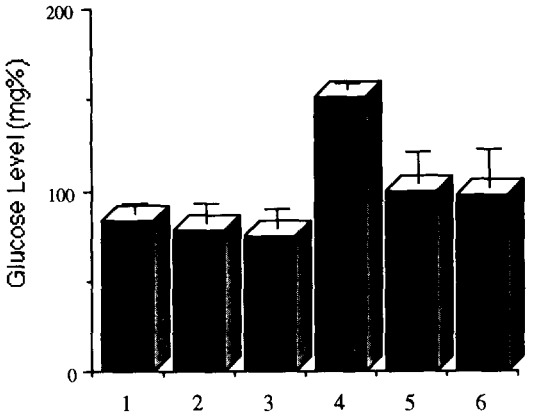

Fig. 2. Effects of PT and mPT on epinephrine-induced hyperglycemia.
Mice received an intravenous injection of either PT (lane 2, 5) or mPT (lane 3, 6) of 250 ng, control mice (lane 1, 4) received normal saline. The blood glucose concentration of each animal was determined immedialely before (lane 1, 2, 3) and 60 min after (lane 4, 5, 6) receiving the epinephrine injection. The results are expressed as mean± SEM of three similary treated mice.

respectively, Ui *et al.*[12] have previously shown that reductive methylation of PT, a procedure which resulted in the methylation of the amino groups of the lysine, markedly diminished the capacity of the toxin to elicit Class II activities while leaving the Class I activities relatively intact.

Experiments were designed to confirm that both PT and mPT were capable of ADP-ribosylating G protein *in vivo* and *in vitro*. As shown in Table I the preincubation of intact macrophages with either PT or mPT resulted in a equivalent reduction of the available endogeneous membrane proteins susceptible to ribosylation with activated PT subsequent to membrane preparations. Experiments were also conducted where preparations of macrophage membranes were incubated with [^{3}H] NAD in the presence of either PT or mPT. As shown in Fig. 1 the treatment of macrophage membrane with either PT or mPT resulted in similar amount of ADP-ribosylation of 41 kDa proteins on the SDS-polyacrylamide gel electrophoresis. These results indicate that both PT and mPT have intact Class I activities.

PT and mPT block epinephrine induced hyperglycemia

Effects of PT and mPT on epinephrine induced hyperglycemia were compared to determine whether PT and mPT could also function *in vivo* to mediate Class I PT activities. Katada and Ui[16,17] have demonstrated that the failure of epinephrine to induce hyperglycemia in pertussis vaccine treated animals resulted from a paradoxical epinephrine induced hyperinsulinemia which was a consequence of the ability of PT to abolish the normal α-adrenergic secretion of pancreatic cells to epinephrine, a Class I effect.

It was determined that einephrine induced hyperglycemia could not be elicited in animals who had received PT or mPT (Fig. 2). These result indicate that mPT was able to block the α-adrenergic response of pancreatic islet cells to epinephrine to the same degree as native PT.

mPT loses the toxin's Class II activities

As the lymphocytosis-promoting action of PT has been shown to represent a Class II effect of the toxin, the effect of methylation of PT on the lymphocytosis-promoting activity was evaluated. As shown in Fig. 3, PT treatment elicits the marked lymphocytosis whereas mPT treatment causes slight elevation of lymphocyte count. These results indi-

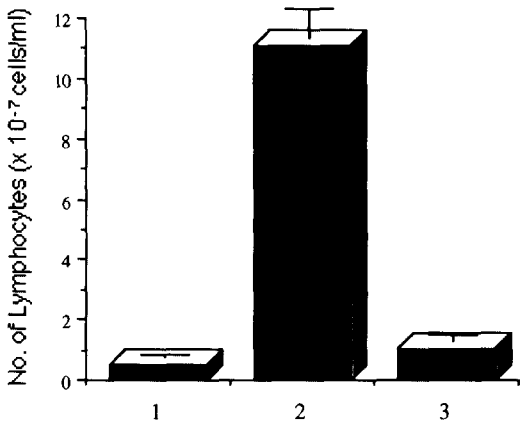

Fig. 3. Lymphocytosis promoting activities of PT and mPT.
Mice received an IV injection of either PT (lane 2) or mPT (lane 3) of 259 ng. Control mice received normal saline (lane 1). Data are expressed as mean± SEM of three similarly treated mice.

cate that methylation of PT attenuates its lymphocytosis-promoting activity, Class II biological activity.

PT can activate murine macrophage but mPT cannot

Having established that mPT has Class I activities but lost its Class II activities, the question of whether macrophage activation with PT is due to the stimulation of Class I or Class II activities of PT was addressed. C_3H/HeN mice were treated with PT or mPT and macrophage NADase was measured as described in "Materials and Methods". Macrophages from PT-treated mice had about 60% NADase activity of resident macrophages of the control mice, whereas the enzyme activity of macrophage from the mPT-treated mice was the same as that of macrophages from the control mice (Table II). The activated macrophages from the lipopolysaccharide treated mice also showed decreased NADase activity. Table III shows the results of experiment in which peritoneal macrophages were incubated with PT or mPT *in vitro*. PT treated cells showed the decrease of NADase activity but mPT-treated cells did not show any changes of the activity. These results suggest that the involvement of PT in the activation of macrophage is not due to its ADP-ribosyl transferase activity. Rather, the action of PT on macrophage activation may be associated with one of Class II activities of the toxin.

Table I. ADP-ribosylation of the membrane of macrophage with PT or methylated PT

Preincubation[a]	The degree of modification(%)[b]
PT	57.0± 1.2
Methylate PT	62.6± 1.6

[a]After macrophages were preincubated for 2 hr at 37℃ either without or with 20 ng/ml of PT or methylated PT, the crude membrane pellets were prepared. Membranes were extracted and subjected to ADP-ribosylation with [^{3}H] NAD in the presence of activated PT (5 μg/ml).
[b]The[^{3}H] ADP-ribosylation reactions were performed in duplicates and the degress of modification(%) was calculated as described under "Materials and Methods".

Table II. Effects of the *in vitro* treatment in mice with PT, methylated PT or LPS on macrophage NADase

Treatment[a]	NADase[b]	
	μmole of cleaved NAD	1% of control
Control	0.101+0.055	
PT	0.060+0.061	59.4
Methylated PT	0.100+0.089	99.0
LPS[c]	0.08+0.026	67.3

[a]Each groups of mice were injected 250 ng of PT or 250 ng of mPT or 10 μg of LPS. Control mice received normal saline.
[b]The activities of macrophage NADase were determined at 96 hr after injection.
[c]LPS was used for positive control to decrease macrophage NADase.

PT can induce the production of acute phase reactants via Class II activity.

Stimulated macrophages have been reported to be a major source of interleukin-1 which enhances the production of acute phase reactants such as surum amyloid-P[17]. Table IV showed interleukin-1 increased production of acute phase reactants. The macrophages from the animal that was treated with PT seemed to generate enough IL-1 to produce serum amyloid-p, sera from the mice treated with mPT contained only low amounts of serum amyloid-P, but treatment of mPT produced only low amounts of serum amyloid-P. These results also indicate that the activation of macrophages is associated with the

Table III. The effect of *in vitro* treatment of PT or methylated PT on macrophage NADase

Treatment[a]	NADase[b] (μmole of cleaved NAD)
Control	0.105+0.059
PT	0.086+0.044
Methylated PT	0.109+0.012

[a]Cells were incubated in RPMI medium containing 50 units/ml penicillin, 50 μg/ml streptomycin and 1 mM sodium pyruvate for 24 hr at 37℃ in the presence of 5% CO_2. PT or mPT was added to the medium at the concentration of 25 μg/ml.
[b]The activities of macrophage NADase were determined 24 hr after incubation with toxins.

Table IV. Effects of PT or methylated PT on the acute phase responses

Treatment[a]	SAP (μg/ml)[b]
Control	5+2
PT	44+3
Methylated PT	15+4
IL-1	98+9

[a]Each groups of mice were injected 250 mg of PT or 250 mg of mPT 32g enzyme units of IL-1. Control mice received normal saline.
[b]Blood was collected at 48 hrs after injection and the plasma analyzed for SAP by radial immunodiffusion.

Class II activity of toxin.

DISCUSSION

In this study evidence was presented that mouse macrophages can be activated by PT; these effects were measured by a decrease of ecto-NADase activity of macrophages. Previously Artman and Seeley[9] demonstrated that activated macrophage by LPS were accompained with a decrease of the activity of NADase.

PT was reductively methylated to modified B-oligomer, but left intact in A-protomer, the macrophages treated with mPT *in vivo* or *in vitro* did not show the decrease of NADase activity, but PT as well as LPS activated the macrophages which have shown the decrease of the enzyme activities. These data suggest that B-oligomer of PT might be responsible for the activation of macrophages.

Ui *et al*[12] classified the biological activities of PT into two classes, Class I and Class II. Class I activities are related to the capacity of the ADP-ribosyl transferase activity of A-protomer of the toxin. Class II activities belong to B-oligomer's activities, which are closely related to immunologic phenomena. Thus, the ability of the toxin to activate the marcrophages may also be classified into Class II activities of the toxin. Mouse macrophages have been known to contain a PLC of high activity and much higher amounts of NADase than that of other tissues[19-21]. Kim *et al*[7] showed that some proportion of erythrocyte NADase from various species is anchored on the membrane surface *via* phosphatidylinositol linkages, although mouse macrophage NADase has not revealed to be through phosphatidylinositol linkage. When macrophages are activated endogeneous PLC would be activated to release the ecto-enzymes, such as NADase or 5′-nucleotidase which has shown to be linked *via* phosphatidylinositol linkages in some cells[7,21]. Endogeneous PLC could be cytosolic or membrane bound ecto-enzyme, but the membrane enzyme may be more plausible to release the ecto-enzyme. This possibility would be supported by the recent findings to Ting and Pagano[22] that one form of PLC is localized at the surface of Swiss 3T3 cells.

In summary, B-oligomer of PT may activate the macrophages, which had shown the decreased activity of NADase. The mechanism of the decrease would be proposed that activated PLC of the macrophages may cleave the phosphatidylinositol-linked ecto-NADase to release, but further studies are required to explain the mechanisms.

LITERATURE CITED

1. Kaplan, N.O.: Animal tissue NADase (Pyridine Transglycosidase). *Methods Enzymol.*, **2**, 660-663 (1955).
2. Swislocki, N.I., Kalish, M.I., Chasalow, F.I. and Kaplan, N.O.: Solubilization and comparative properties of some mammalian diphosphopyridine nucleosides. *J. Biol. Chem.*, **242**, 1089-1094 (1967).
3. Srivastava, S.K., Mini, S.B. and Ramakrishiman, C.V.: Studies on NAD glycohydrolase in Rumex acetosa tumor tissue cultivated *in vitro*. *Phytochemistry*, **8**, 1147-1154 (1965).

4. Dewolf, M. J. S., Van Dessel, G. A. F., Hilderson, H. J. J. and Dierick, W. S. H., Topography, purification and characterization of thyroidal NAD^+ glycohydrolase. *Biochem. J.*, **226**, 415-427 (1985).
5. Pekala, P. H. and Anderson, B. M.: Studies of bovine erythrocyte NAD glycohydrolase. *J. Biol. Chem.*, **253**, 7453-7459 (1978).
6. Friedemann, H. and Rapotot, S. M.: Enzymes of the red cell: *A critical catalogue in cellular and molecular biology of erythrocytes* (Yoshikawa, H. and Rapopot, S. M., eds), 181-259, University Park Press. Baltimore. (1974).
7. Kim, U. H., Rookwood, S. F., Kim, H. R. and Daynes.R. A.: Membrane-associated NAD^+ glycohydrolase from rabbit erythrocytes is solubilized by phosphatidylinositol specific phospholipase C. *Biochim. Biophys. Acta.*, **965**, 76-81 (1988).
8. Artman, M., Seeley, R. J. and Ishii, S.: Nicotinamide adenine dinucleotide splitting enzyme: A plasma membrane protein of murine macrophages. *Arch. Biochem. Biophys.*, **195**, 121-127 (1979).
9. Artman, M. and Seely, R. J.: Nicotinamide adenine dinucleotide splitting enzyme in normal, elicited and activated peritoneal macrophages of the mouse. *J. Reticuloendothel. Soc.*, **25**, 479-487 (1979).
10. Tamura, M. and Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada,T. and Ui, M.: Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry*, **21**, 5516-5522 (1982).
11. Katada, T., Tamura, M. and Ui, M.: The a protomer of islet activating protien, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch. Biochem. Biophys.* **224**, 290-298 (1985).
12. Ui, M., Nogimori, K. and Tamura, M.: Islet activating protein, pertussis toxin: Subunit structure and mechanism for its multiple biological actions. Sekura, R. D., Moss, J., Vaughn, M. (eds), *Pertussis toxin*, pp. 19-43, 1985, Academic Press (1985).
13. Yajima, M., Josoda, K., Kambayashi, Y., Nakamura, T., Takahashi, T. and Ui, M.: Biological properties of islet activating protein (IAP) purified from the culture medium of Bordetella pertussis. *J. Biochem.*, **83**, 305-312 (1978).
14. Kunst, A., Draeger, B. and Ziegenhorn, J.: Colorimetric methods with glucose oxidase and peroxidase. *Method of Enzymatic Anal.* **6**, Verlag Chemie pp. 178-185 (1984).
15. Backnd, P. S., Mead, B. D., Manclark, C. R., Centoni, G. L., Akasamit and R. R.: Pertussis toxin inhibition of chemotaxis and the ADP-ribosylation of a membrane protein in a human-mouse hybrid cell line. *Proc. Natl. Acad. Sci. USA.*, **82**, 2637-2641 (1985).
16. Katada, T. and Ui, M.: Accelerated turnover of blood glucose in pertussis-sensitized rats due to combined actions of endogenous insulin and adrenergic beta-stimulation. *Biochim. Biophys. Acta.* **421**, 57-69 (1976).
17. Ui, M., Katsumi, N. and Makoto, T.: Islet-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions. *Pertussis toxin*, Sekura R. D., Moss, J. and Vaughan, M. (eds). Acadamic Press, Orland (1985).
18. Dinarello, C. A.: Induction of acute phase reactants by interleukin-1. *Adv. Inflammation Res.*, **8**, 203-225 (1984).
19. Wighthman, P. D., Dahlgren, M. E., Hill, J. C., Davies, P. and Bonney, R. J.: Identification and characterization of a phospholipase C activity in resident mouse peritoneal macrophages. *Biochem. J.*, **197**, 523-526 (1981).
20. Artman, M. and Seely, R. J.: Nicotinamide adenine dinucleotide splitting enzyme: A characteristic of the mouse macrophage. *Science*, **202**, 1923-1925 (1978).
21. Low, M. G. and Fenean, J. B.: Specific release of plasma membrane enzymes by a phosphatidylinositol-specific phospholipase C. *Biochim. Biophys. Acta.*, **508**, 565-570 (1978).
22. Ting, A. E. and Phgano, R. E.: Detection of a phosphatidyl specific phospholipase C at the surface of Swiss 3T3 cells and its potential role in the regulation of cell growth. *J. Biol. Chem.*, **265**, 5337-5340 (1990).