INFECTION AND IMMUNITY, Mar. 1997, p. 1114–1118 Vol. 65, No. 3
0019-9567/97/$04.00+0

Role of Pertussis Toxin A Subunit in Neutrophil Migration and Vascular Permeability

GERLY A. C. BRITO,[1] MARCELLUS H. L. P. SOUZA,[1] ANTÔNIO A. MELO-FILHO,[1] ERIK L. HEWLETT,[2] ALDO A. M. LIMA,[1] CARLOS A. FLORES,[1] AND RONALDO A. RIBEIRO[1]*

Department of Physiology and Pharmacology, Clinical Research Unit-HUWC, Federal University of Ceará, Fortaleza, Ceará, Brazil,[1] and Division of Clinical Pharmacology, School of Medicine, University of Virginia, Charlottesville, Virginia[2]

Received 7 August 1996/Returned for modification 3 October 1996/Accepted 9 December 1996

The anti-inflammatory activity of pertussis toxin (Ptx) was compared to that of a noncatalytic mutant of pertussis toxin (9K/129G; Ptxm), which contains two amino acid substitutions in the A protomer, by using a rat model of inflammation. The toxins were administered intravenously 1 h prior to the injection of inflammatory stimuli. Ptx, but not Ptxm, inhibited neutrophil migration into peritoneal cavities in response to formyl-methionyl-leucyl-phenylalanine and lipopolysaccharide. The inhibitory effect of Ptx on neutrophil migration could not be explained by the ability of the toxin to induce leukopenia or neutropenia. The increase in skin vascular permeability induced by leukotriene B_4, a powerful neutrophil chemotactic agent, was also inhibited only by Ptx. On the other hand, the increase in skin vascular permeability induced by histamine was potentiated by both toxins. These data show that Ptx inhibits neutrophil-mediated inflammation in vivo and that this effect is dependent on the ADP-ribosyltransferase activity of the A protomer.

Pertussis toxin (Ptx) is an exotoxin produced by *Bordetella pertussis*, the bacterium that causes whooping cough. Ptx is a hexameric protein with typical AB architecture, consisting of an A protomer (S1) and a B oligomer (S2, S3, two S4s, and S5). The A protomer has ADP-ribosyltransferase activity, which catalyzes the transfer of ADP-ribose from NAD to a regulatory GTP-binding protein (G protein) in eukaryotic cells. The B oligomer also has some biological activities, such as the activation of platelets and mitogenicity for lymphocytes, but these are independent of the enzymatic activity of the A protomer (1, 27).

Recently, we reported that the intravenous (i.v.) administration of Ptx blocked the migration of neutrophils into rat peritoneal cavities injected with *Escherichia coli* lipopolysaccharide (LPS) and formyl-methionyl-leucyl-phenylalanine (fMLP). Ptx also reduced neutrophil-mediated edema, but not the edema dependent on basophil-mast cell degranulation (28). In addition, Ptx has been shown to inhibit monocyte/macrophage migration in vitro and in vivo (11, 12). These observations suggest that the effect of Ptx on leukocyte migration is probably due to a direct action on these cells. The lectin domains of the S2 and S3 subunits of Ptx share amino acid sequence homology with the lectin domains of selectins (25), a family of glycoproteins that mediate leukocyte-endothelial cell interactions in the early stages of leukocyte transendothelial migration (9, 10). The B oligomer, the individual S2 and S3 subunits, or peptides derived from the S2 and S3 lectin domains are able to inhibit leukocyte adherence to selectin-coated surfaces in vitro (23), as well as leukocyte recruitment into cerebrospinal fluid after pneumococcal challenge in vivo (24). Thus, although the mechanisms involved in the inhibition of leukocyte migration by Ptx are not completely understood, the A protomer and/or the B oligomer appears to be involved.

* Corresponding author. Mailing address: Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde, Universidade Federal do Ceará, Rua Cel Nunes de Melo, 1127, 60.430-270 Fortaleza, Ceará, Brazil. Phone: 55-85-243-9349. Fax: 55-85-243-9333.

In the present work, we have determined which subunit of Ptx is responsible for its anti-inflammatory effect in vivo by using a site-directed mutant toxin, 9K/129G (Ptxm). This toxin has been used as a nontoxic Ptx in immunization protocols (8, 19, 21). Ptxm was prepared at Chiron Bioscine (Siena, Italy) and was kindly provided by Rino Rappuoli. This mutant contains two amino acid substitutions in the S1 subunit (substitutions of lysine for arginine at position 9 [Arg9→Lys] and glycine for glutamic acid at position 129 Glu129→Gly), which abolish the subunit's enzymatic activity (15, 19). The wild-type toxin (Ptx) was purified from the supernatants of *B. pertussis* 165 cultures by hydroxylapatite chromatography and fetuin affinity chromatography and stored at 4°C (26). The anti-inflammatory activity was evaluated in the classical Wistar rat model of peritonitis (28) following stimulation with LPS and fMLP. The increase in skin vascular permeability was measured as the extravasation of Evans blue dye (22, 31) in response to leukotriene B_4 (LTB_4) and histamine.

The i.v. administration of Ptx (200 or 1,200 ng/rat) 1 h before LPS (from *E. coli* O111:B4; Difco, Detroit, Mich.; 200 ng) (Fig. 1A) or fMLP (Sigma Chemical Co., St. Louis, Mo.; 44 ng) (Fig. 1B) challenge reduced neutrophil migration into rat peritoneal cavities in a dose-dependent manner. On the other hand, Ptxm, even at a dose of 1,200 ng/rat, did not alter the fMLP- or LPS-induced neutrophil migration into peritoneal cavities (Fig. 1). LPS-induced peritonitis seemed to be more sensitive to Ptx since 75 ng of this toxin was effective in reducing the neutrophil migration induced by the endotoxin but not that induced by fMLP (data not shown). The anti-inflammatory effect of Ptx was not attributable to the induction of neutropenia since the i.v. administration of 200 ng of Ptx did not change the leukogram of the animals within 5 h after injection. After 3 days, Ptx induced leukocytosis and neutrophilia (Fig. 2). Ptx also increased the number of circulating lymphocytes and monocytes (data not shown), a classical phenomenon known to be caused by this toxin (13). At the same doses as Ptx, Ptxm did not alter the total or differential counts of rat peripheral blood leukocytes (Fig. 2). Findings similar to those de-

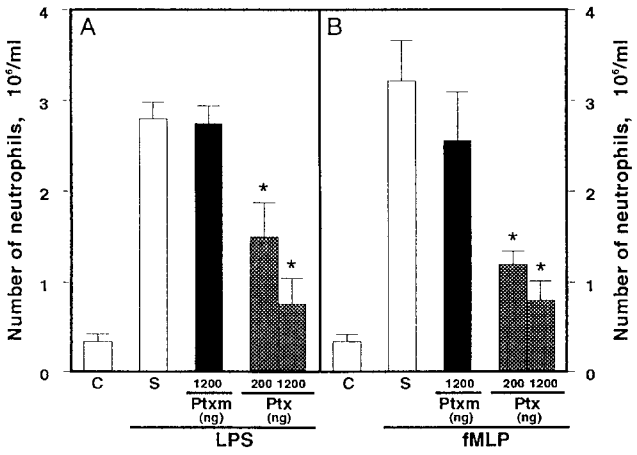

FIG. 1. Wild-type (Ptx) but not mutant (Ptxm) pertussis toxin inhibits LPS- (A) and fMLP (B)-induced neutrophil migration into the rat peritoneal cavity. The results are the means ± the standard errors of the means of data for six rats per group; *, $P < 0.05$ in relation to the group that received saline i.v. (S; positive control) by analysis of variance-Tukey.

scribed above have been described by Meade et al. (12) and by Nencioni et al. (15, 16) for Ptx and Ptxm, respectively. Thus, the Ptx-induced alterations in circulating leukocyte numbers are also dependent on the catalytic activity of the A protomer. These results corroborate the clinical findings of high-level lymphocytosis in patients with whooping cough and suggest that this effect is probably due to the presence of Ptx in the circulatory systems of *B. pertussis*-infected patients (18). The effect of 1,200 ng of Ptx or Ptxm on LPS-induced neutrophil migration into peritoneal cavities was also studied by histopathological analysis. The LPS-inflamed mesentery was removed, fixed in 10% neutral buffered formalin, sectioned into small pieces, and mounted directly on albumin-coated glass slides prior to staining with hematoxylin and eosin. The neutrophil infiltration in the inflamed mesentery 4 h after toxin injection was not intense in Ptx-treated animals, and these cells were retained in the lumen of the mesenteric blood vessels (Fig. 3E and F). On the other hand, the neutrophil infiltrate in the LPS-inflamed mesentery of the Ptxm-treated rat was intense and diffuse (Fig. 3C and D), and no differences were observed upon comparison with the control group (Fig. 3A and B). In agreement with these findings, we observed that Ptx caused a less intense (30%) but still significant reduction in carrageenin-induced rat paw edema. On the other hand, Ptx had no effect on dextran-induced paw edema, a typical neutrophil-free edematogenic reaction. Ptxm was ineffective in both of these experimental models (data not shown). These results confirm and extend our previous work (28) by showing that Ptx inhibits fMLP- and LPS-induced neutrophil migration into the rat peritoneal cavity and also inhibits neutrophil-mediated inflammatory reactions. As Ptxm was devoid of such actions, we conclude that ADP-ribosyltransferase activity is essential for the in vivo anti-inflammatory action of Ptx.

Leukocyte recruitment in vivo is largely dependent on the expression of adhesion molecules at the cell surface (9, 10, 33). As mentioned above, the S2 and S3 subunits, or peptides from their lectin domains, inhibit neutrophil adherence in vitro (23) and leukocyte migration in vivo (24). These effects were explained by assuming that there is a competitive blockage of the eukaryotic selectin carbohydrate-specific binding sites. However, we have demonstrated here that Ptxm has no anti-inflammatory activity in any of the models tested despite the fact that it possesses the same B oligomer structure as Ptx (19, 32). The lack of activity in Ptxm cannot be ascribed to a low dosage since the toxin was administered in the same dose range as that reported for S2 and S3 (24). In addition, the histopathology of the inflamed mesentery showed clearly that Ptxm had no inhibitory effect on LPS-induced neutrophil migration, even at a dose 16-fold higher than the lowest effective dose for the wild-type toxin. These contradictory findings could reflect the fact that we used the whole toxin instead of the isolated B subunit or peptides from the S2 and S3 lectin domains. The interection of the Ptx S2 and S3 subunits with human leukocytes is preferentially inhibited by galactose and sialylated glycoconjugates, respectively (30). Sialosyl-Lewis-x, the most important sialylated glycoconjugate in humans, is expressed at a high level in human neutrophils and is considered to be the binding site for selectins (6). However, since this epitope is highly species specific and is absent in nonhuman mammalian species, it cannot be considered to be a general binding site for interaction with selectins in nonhuman leukocytes (6).

We also studied the effect of Ptx and Ptxm (1,200 ng/rat, i.v.) on the increase in rat skin vascular permeability induced by LTB_4 (Sigma Chemical Co.), a neutrophil chemotactic mediator, and histamine (Sigma Chemical Co.), a vasoactive agent, coinjected with 200 ng of prostaglandin I_2 (Sigma Chemical Co.). One hour after LTB_4 administration (2 ng/site) or im-

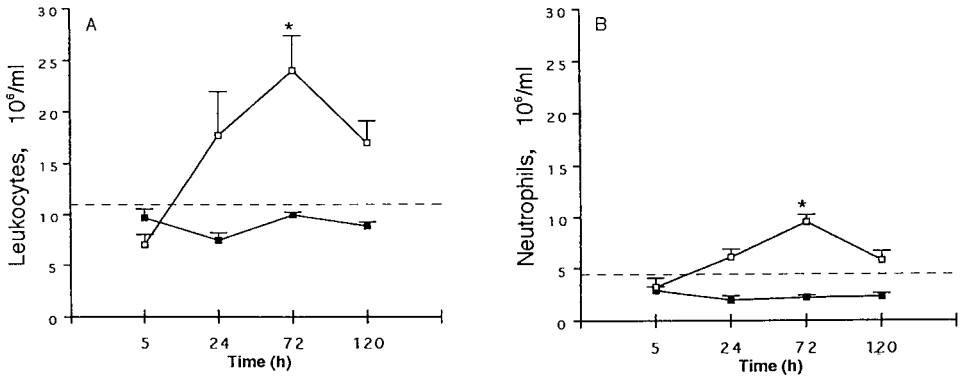

FIG. 2. The effect of wild-type (Ptx) (□) or mutant (Ptxm) (■) pertussis toxin on the total leukocyte (A) and neutrophil (B) counts in peripheral blood of normal animals. The mean values for leukocytes and neutrophils in phosphate-buffered saline (PBS)-treated rats (control, dashed line; $n = 4$) were 11×10^6 cells/ml and 4.3×10^6 cells/ml, respectively. The amount of Ptx and Ptxm injected was 200 ng/rat. Each point represents the mean ± the standard error of the mean of the data from four animals; *, $P < 0.05$ in relation to the PBS (control) group by analysis of variance for repeated measurements.

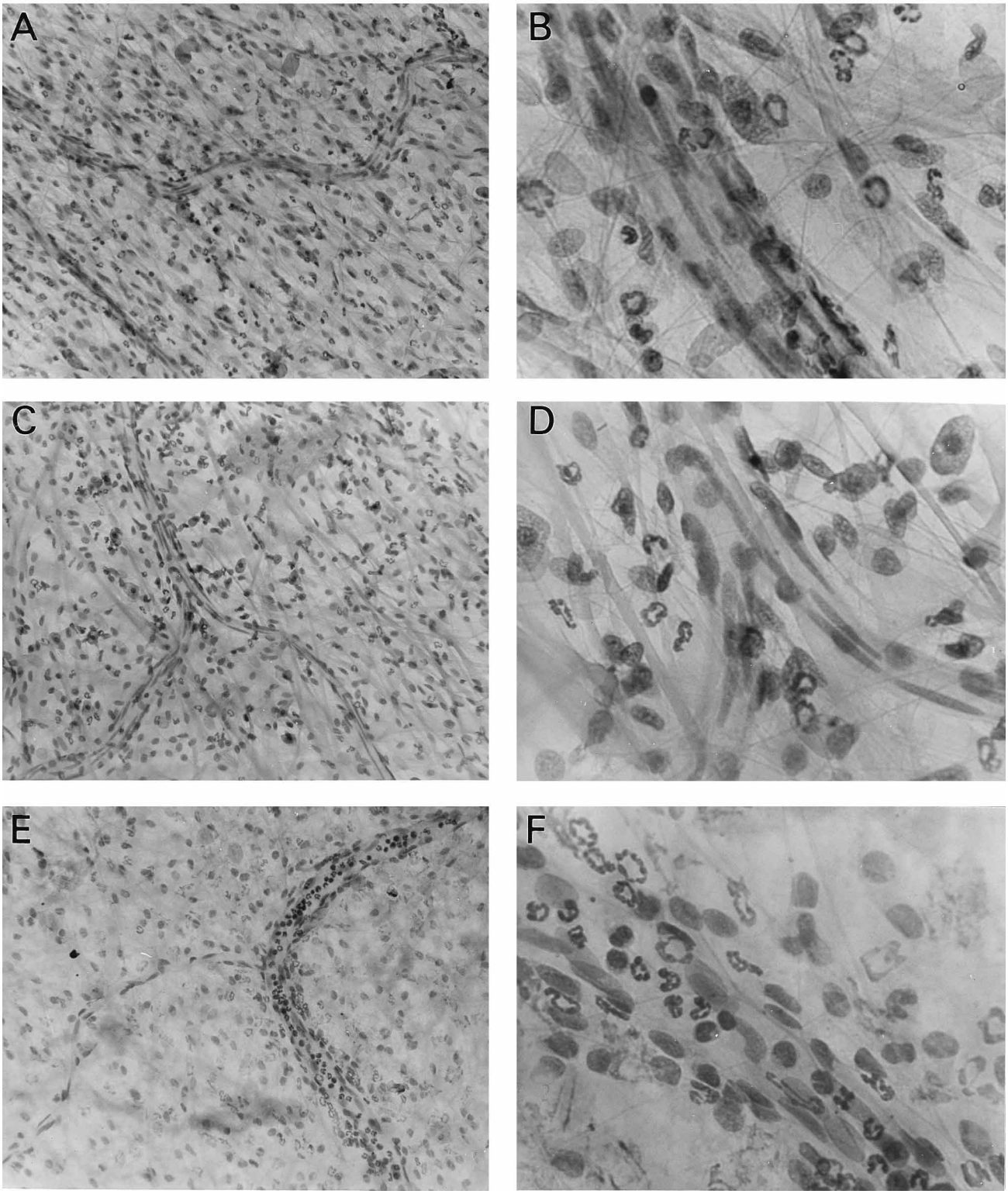

FIG. 3. Histological appearance of inflamed mesenteries of rats injected i.p. with LPS (200 ng/cavity) and pretreated i.v. with sterile saline (A and B) or with 1,200 ng of mutant (Ptxm) (C and D) or wild-type (Ptx) (E and F) pertussis toxin per animal. The neutrophil infiltrate in the Ptx-treated rats was clearly less intense than that observed in the saline- or Ptxm-treated groups. In the latter animals, an intense, diffuse neutrophil infiltrate was observed. In Ptx-treated rats, the neutrophils were retained within the blood vessels. These sections were obtained 4 h after treatment with LPS. Hematoxylin and eosin staining is shown. Magnifications, ×100 (A, C, and E) and ×400 (B, D, and F).

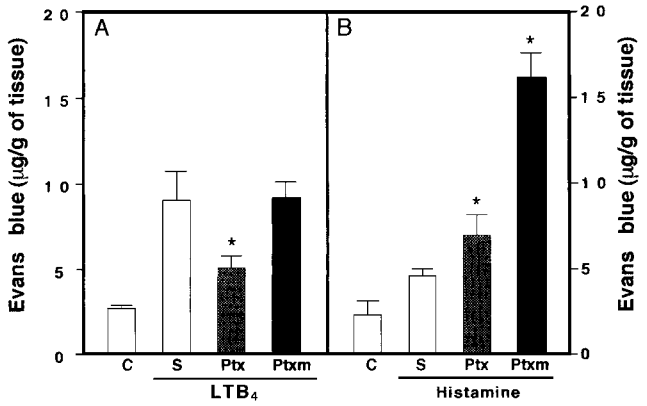

FIG. 4. The effect of wild-type or mutant (Ptxm) pertussis toxin on Evans blue dye extravasation induced by a subcutaneous injection of LTB_4 (2 ng/site) (A) or histamine (10 μg/site) (B). The results represent the means ± the standard errors of the means of the data for six determinations per stimulus, in at least three different animals; *, $P < 0.05$ in relation to the group that received saline i.v. (S; positive control) by analysis of variance-Tukey.

mediately after histamine injection (10 μg/site), the animals received Evans blue dye (Aldrich Chemical Co., Milwaukee, Wis.) (25 mg/kg, i.v., 100 μl/100 g of body weight). The LTB_4 and histamine groups were sacrificed 1 h and 30 min, respectively, after the vital-dye injection. Pretreatment with Ptx diminished (by 45%; $P < 0.05$) the increase in vascular permeability induced by LTB_4, while Ptxm, at the same dose, had no effect on the dye extravasation (Fig. 4A). The increase in vascular permeability induced by histamine was potentiated ($P < 0.05$) by both toxins (Fig. 4B). The histamine-sensitizing capacity of Ptx is a well-known event (7), and the present data demonstrate that the B oligomer contributes substantially to this effect, since the potentiating action of Ptxm was twofold higher than that of Ptx. This result agrees with previous work showing that the histamine-potentiating action of Ptx is mediated by the S3 and S4 subunits (29), even though Ptxm failed to sensitize mice to a histamine challenge (16, 21).

In conclusion, we have provided evidence that Ptx is effective only in neutrophil-mediated inflammatory reactions. There is substantial evidence that various chemotactic mediators (e.g., fMLP, interleukin-8, platelet-activating factor, and C5a) cause neutrophil migration by acting on specific cell receptors belonging to the superfamily of G protein-coupled receptors (see references 3 and 14 for reviews). The conceivable signal transduction sequence implicated in stimulus-induced leukocyte chemotaxis involves receptor-ligand binding, activation of a Ptx-sensitive trimeric G protein, phospholipase C activation, increases in intracellular inositol 1,4,5-triphosphate and Ca^{2+} levels, and F actin formation (2, 3, 4, 15). Thus, we propose that the anti-inflammatory effect of Ptx in vivo is dependent on the enzymatic activity of the A protomer subunit and on the interaction of the latter with G proteins involved in signal transduction following the stimulation of leukocytes by chemotactic agents. In support of this, we have shown that Ptxm, which is devoid of enzymatic activity, also has no anti-inflammatory effect.

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 52.1015/94) and Fundação Cearense de Amparo à Pesquisa (FUNCAP; grant SOS 092/94).

We thank Rino Rappuoli for kindly provided the toxins and Stephen Hyslop for language review.

REFERENCES

1. **Banga, H. S., R. K. Walker, L. K. Winberry, and S. E. Rittenhouse.** 1987. Pertussis toxin can activate human platelets. J. Biol. Chem. **262:**14871–14874.
2. **Becker, E. L., J. C. Kermode, P. H. Naccache, R. Yassin, J. J. Munoz, M. L. Marsh, C. K. Huang, and R. I. Sha'afi.** 1986. Pertussis toxin as a probe of neutrophil activation. Fed. Proc. **45:**2151–2155.
3. **Ben-baruch, A., D. F. Michiel, and J. J. Oppenheim.** 1995. Signals and receptors involved in recruitment of inflammatory cells. J. Biol. Chem. **270:** 11703–11706.
4. **Bengtsson, T., E. Sarndahl, O. Stendahl, and T. Andersson.** 1990. Involvement of GTP-binding proteins in action polymerization in human neutrophils. Proc. Natl. Acad. Sci. USA **87:**2921–2925.
5. **Fruman, D. A., D. A. Gamache, and M. J. Ernest.** 1991. Changes in inositol 1,4,5-trisphosphate mass in agonist-stimulated human neutrophils. Agents Actions **34:**16–19.
6. **Hakomori, S., and I. Yasuyuki.** 1995. Functional role of glycosphingolipids in cell recognition and signaling. J. Biochem. **118:**1091–1103.
7. **Linthicum, D. S., J. J. Munoz, and A. Blaskett.** 1982. Acute experimental autoimmune encephalomyelitis in mice. I. The adjuvant action of *Bordetella pertussis* is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. Cell. Immunol. **73:**289–310.
8. **Marsili, I., M. Pizza, F. Giovannoni, G. Volpini, M. Bartalini, R. Olivieri, R. Rappuoli, and L. Nencioni.** 1992. Cellular pertussis vaccine containing a *Bordetella pertussis* strain that produces a nontoxic pertussis toxin molecule. Infect. Immun. **60:**1150–1155.
9. **McEver, R. P.** 1991. Leukocyte interactions mediated by selectins. Thromb. Haemostasis **66:**80–87.
10. **McEver, R. P.** 1992. Leukocyte-endothelial cell interactions. Curr. Biol. **4:**840–849.
11. **Meade, B. D., P. D. Kind, J. B. Ewell, P. P. McGrath, and C. R. Manclark.** 1984. In vitro inhibition of murine macrophage migration by *Bordetella pertussis* lymphocytosis-promoting factor. Infect. Immun. **45:**718–725.
12. **Meade, B. D., P. D. Kind, and C. R. Manclark.** 1984. Lymphocytosis-promoting factor of *Bordetella pertussis* alters mononuclear phagocyte circulation and response to inflammation. Infect. Immun. **46:**733–739.
13. **Munoz, J. J., H. Arai, and R. K. Bergman, and P. L. Sadowski.** 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect. Immun. **33:**820–826.
14. **Murphy, P. M.** 1994. The molecular biology of leukocyte chemoattractant receptors. Annu. Rev. Immunol. **12:**593–633.
15. **Nencioni, L., M. Pizza, G. Volpini, M. T. DeMagistris, F. Giovannoni, and R. Rappuoli.** 1991. Properties of the B oligomer of pertussis toxin. Infect. Immun. **59:**4732–4734.
16. **Nencioni, L., M. Pizza, M. Bugnoli, T. DeMagistris, A. Di Tommaso, F. Giovannoni, R. Manetti, I. Marsili, G. Matteucci, D. Nucci, R. Olivieri, P. Pileri, R. Presentini, L. Villa, J. G. Kreeftenberg, S. Silvestri, A. Tagliabue, and R. Rappuoli.** 1990. Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. Infect. Immun. **58:**1308–1315.
17. **Ohta, H., F. Okajima, and M. Ui.** 1985. Inhibition by islet-activating protein of a chemotactic peptide-induced early breakdown. J. Biol. Chem. **260:** 15771–15780.
18. **Olson, L. C.** 1975. Pertussis. Medicine **54:**427–469.
19. **Pizza, M., A. Covacci, A. Bartoloni, M. Perugine, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, O. Roberto, J. T. Barbieri, H. Sato, and R. Rappuoli.** 1989. Mutants of pertussis toxin suitable for vaccine development. Science **246:**497–500.
20. **Rankin, J. A., I. Sylvester, S. Smith, T. Yoshimura, and J. Leonard.** 1990. Macrophages cultured *in vitro* release leukotriene B_4 and neutrophil attractant/activation protein (interleukin 8) sequentially in response to stimulation with lipopolysaccharide and zymosan. J. Clin. Invest. **86:**1556–1564.
21. **Rappuoli, R., A. Podda, M. Pizza, A. Covacci, A. Bartoloni, M. T. De Magistris, and L. Nencioni.** 1992. Progress toward the development of new vaccines against whooping cough. Vaccine **10:**1027–1032.
22. **Reis, M. L., L. Okino, and M. Rocha e Silva.** 1973. Bradykinin derivatives as permeability factor. Agents Actions **3:**388–392.
23. **Rozdzinski, E., W. N. Burnette, T. Jones, V. Mar, and E. Tuomanen.** 1993. Prokaryotic peptides that block leukocyte adherence to selectins. J. Exp. Med. **178:**917–924.
24. **Rozdzinski, E., T. Jones, W. N. Burnette, M. Burroughs, and E. Tuomanen.** 1993. Antiinflammatory effects in experimental meningitis of prokaryotic peptides that mimic selectins. J. Infect. Dis. **168:**1422–1428.
25. **Saukkonen, K., W. N. Burnette, V. Mar, H. R. Masur, and E. Tuamanen.** 1992. Pertussis toxin has eukaryotic-like carbohydrate recognition domains. Proc. Natl. Acad. Sci. USA **89:**118–122.
26. **Sindt, K. A., E. L. Hewlett, G. T. Redpath, R. Rappuoli, L. S. Gray, and S. R. Vandenberg.** 1994. Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. Infect. Immun. **62:**3108–3314.
27. **Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii.** 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry **21:**5516–5522.

28. **Thomazzi, S. M., M. H. L. P. Souza, A. A. Melo-Filho, E. L. Hewlett, A. A. M. Lima, and R. A. Ribeiro.** 1995. Pertussis toxin from *Bordetella pertussis* blocks neutrophil migration and neutrophil-dependent edema in response to inflammation. Braz. J. Med. Biol. Res. **28:**120–124.
29. **Ui, M., K. Nogimore, and M. Tamura.** 1985. Islet-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions, p. 19–43. *In* R. D. Sekura, J. Moss, and M. Vaughn (ed.), Pertussis toxin. Academic Press, New York, N.Y.
30. **van't Wout, J., W. N. Burnette, V. L. Mar, E. Rozdzinski, S. D. Wright, and E. I. Tuomanen.** 1992. Role of carbohydrate recognition domains of pertussis toxin in adherence of *Bordetella pertussis* to human macrophages. Infect. Immun. **60:**3303–3308.
31. **Wedmore, C. V., and T. J. Williams.** 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature **289:** 646–650.
32. **Zealey, G. R., S. M. Loosmore, R. K. Yacoob, S. A. Cockle, A. B. Herbert, L. D. Miller, N. J. Mackay, and M. H. Klein.** 1992. Construction of *Bordetella pertussis* strains that overproduce genetically inactivated pertussis toxin. Appl. Environ. Microbiol. **58:**208–214.
33. **Zimmerman, G. A., S. M. Prescott, and T. M. McIntyre.** 1992. Endothelial cell interactions with granulocytes: tethering and signaling molecules. Immunol. Today **13:**93–99.

Editor: A. O'Brien