The Inhibition of Neutrophil Granule Enzyme Secretion and Chemotaxis by Pertussis Toxin

E. L. BECKER,* J. C. KERMODE,* P. H. NACCACHE,* R. YASSIN,* M. L. MARSH,* J. J. MUNOZ,‡ and R. I. SHA'AFI*

*Departments of Pathology and Physiology, University of Connecticut Health Center, Farmington, Connecticut 06032; and ‡National Institute of Allergy and Infectious Disease, National Institutes of Health Rocky Mountain Laboratory, Hamilton, Montana 59840

ABSTRACT Pertussis toxin treatment of rabbit peritoneal neutrophils causes a concentration-dependent inhibition of granule enzyme secretion induced by formylmethionyl-leucyl-phenylalanine, C5a, and leukotriene B_4. It also inhibits chemotaxis induced by formylmethionyl-leucyl-phenylalanine. The same toxin treatment, however, has no effect on granule enzyme secretion induced by the calcium ionophore A23187 or phorbol 12-myristate 13-acetate. Moreover, pertussis toxin treatment does not affect either the number or affinity of the formylpeptide receptors on the neutrophil nor does it have any effect on the unstimulated levels of cyclic AMP (cAMP) or the transient rise in cAMP induced by chemotactic factor stimulation in these cells.

We hypothesize that pertussis toxin, as in other cells, interacts with a GTP binding regulatory protein identical with or analogous to either N_i or transducin which mediates the receptor-induced inhibition or activation of a target protein or proteins required in neutrophil activation. The nature of the target protein is unknown, but it is not the catalytic unit of adenylate cyclase. The target protein acts after binding of chemotactic factor to its receptor in the sequence that leads to the receptor-induced rise in intracellular Ca^{2+}. It does not affect the responses elicited by the direct introduction of calcium into the cells or the activity of protein kinase C.

Two GTP binding regulatory proteins, $N_s(G_s)$ and $N_i(G_i)$, mediate the action of a number of hormones and other agents on adenylate cyclase. The proteins involved have been purified and studied in detail (1). Cholera toxin ADP-ribosylates and activates N_s (2). Pertussis toxin ADP-ribosylates and inactivates the inhibitory activity of N_i and the ability of transducin to mediate the light-induced activation of cyclic GMP (cGMP) phosphodiesterase in the retina (3, 4). Pertussis toxin has been crystallized (5, 6) and purified to homogeneity (4). Several observations suggest that GTP regulatory proteins may be involved in neutrophil activation. N_s has been demonstrated in neutrophils (7), and incubation of neutrophils with cholera toxin increases their levels of cyclic AMP (cAMP) and inhibits their chemotactic responsiveness (8, 9). It has been reported that nonhydrolyzable GTP analogues shift the affinity of the formylpeptide receptors of neutrophils (10), and that the chemotactic peptide *N*-formyl-norleucyl-leucyl-phenylalanyl-leucyl-tyrosyl-lysine stimulates the GTPase activity of human neutrophil homogenates (11).

We therefore have tested the effect of pretreatment of rabbit peritoneal neutrophils with crystalline pertussis toxin on granule enzyme secretion and chemotaxis induced by the chemotactic factors, formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe)[1], the fragment of the fifth component of complement (C5a), and the arachidonic acid metabolite, leukotriene B_4 (LTB_4), as well as on their stimulation by the calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA). As we shall show, pretreatment with pertussis toxin inhibits chemotactic factor-induced chemotaxis and granule enzyme secretion from the neutrophil but has no effect on enzyme release caused by PMA or A23187. No detectable effect of toxin treatment is evident on the binding

[1] *Abbreviations used in this paper*: C5a, the fragment of the fifth component of complement; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; fNle-Leu-Phe, formylnorleucyl-leucyl-phenylalanine; LTB_4, leukotriene B_4 [5(S), 12(R)-dihydroxy-6, 14-*cis* 8, 10 *trans* eicosatetraenoic acid]; N_i and N_s, two GTP binding regulatory proteins; PMA, phorbol 12-myristate-13 acetate.

of radiolabeled formylpeptides to their receptor or on the levels of neutrophil cAMP.

MATERIALS AND METHODS

Chemicals: The synthetic peptides fMet-Leu-Phe and formylnorleucyl-leucyl-phenylalanine (fNle-Leu-Phe), A23187, 2 × crystalline ovalbumin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, and shellfish glycogen were obtained from Sigma Chemical Co. (St. Louis, MO). The radiolabeled peptides, fMet-Leu-[^{3}H]Phe (48 Ci/mmol), fNle-Leu-[^{3}H]Phe (42 Ci/mmol), and [^{3}H]cAMP (37 Ci/mmol) were obtained from New England Nuclear (Boston, MA). CMC Cancer Research Chemicals was the source of the PMA, and LTB_4 was a gift from Dr. P. Borgeat (Groupe de Recherches sur les Leucotrienes, Centre Hospitalier de l'Universite Laval, Sainte-Foy, Quebec, Canada). Highly purified C5a was a generous gift from Dr. P. M. Henson (National Jewish Hospital and Research Center, Denver, CO). The crystalline pertussis toxin was prepared and dissolved as described (5).

Rabbit peritoneal neutrophils obtained 4–12 h after the injection of 200–400 ml of sterile 0.1% shell fish glycogen were washed and treated as described (12). For granule enzyme secretion, neutrophils were suspended at a concentration of 5 × 10^6 cells/ml in Mg^{2+}-free modified Hanks' buffer with 1 mg/ml ovalbumin. They were incubated at 37°C with or without the indicated concentration of pertussis toxin; at the end of the indicated times, 0.94 ml of cell suspension was added to 50 μl cytochalasin B and 10 μl of the stimulating compound, and the mixture was incubated for 5 min more. For chemotaxis, 2.5 × 10^6 cells/ml were incubated at 37°C for 1 h with or without toxin, and the chemotactic response was measured as indicated below.

Granule Enzyme Release: Granule enzyme release in the presence of 5 μg/ml of cytochalasin B was performed as previously described (12) and monitored by the assay of lysozyme and β-glucosaminidase. The lysozyme activity was determined by measuring the change in optical density of a suspension of *Micrococcus lysodeikticus* as described previously (12). β-Glucosaminidase was measured by the breakdown of its substrate *p*-nitrophenyl-β-*N*-acetyl glucosaminide as described (13), except that a modification by D. L. Kreutzer was followed in which a 300-μl aliquot was placed in a 96-well microtiter plate after the reaction was stopped, and the optical density was then determined at 410 nm with a Titertek Multiskan apparatus (Flow Laboratories, Inc., McLean, VA). The release of the two enzymes paralleled each other so that only the results for lysozyme are reported. The cells were also tested for lactic dehydrogenase leakage (12). No increase was found even after a 90-min incubation at 37°C with pertussis toxin; therefore, lactate dehydrogenase results are not reported.

Chemotaxis: Chemotaxis was carried out in a multichamber chemotaxis apparatus at 37°C for 1 h using 3-μm pore size filters, as described, except the chambers were not centrifuged (14). The mean number of cells penetrating below the upper monolayer in five high-power fields (magnification of 400) was averaged over duplicate filters as a measure of the chemotactic response.

Receptor Binding: Studies of the binding of fMet-Leu-[^{3}H]Phe and fNle-Leu-[^{3}H]Phe to rabbit peritoneal neutrophils were performed by use of a modification of the silicone oil assay of Mackin et al. (15). In brief, neutrophils obtained 4 h after injection of glycogen were washed twice with Mg^{2+}-free, Ca^{2+} (1.7 mM)-containing Hanks' buffer and resuspended at 5 × 10^6 cells/ml in the same buffer that contained 1 mg/ml crystalline bovine serum albumin (Sigma Chemical Co.). Cells preincubated for 1 h at 37°C in the absence (control cells) or presence of 500 ng/ml pertussis toxin were centrifuged and resuspended to either 4 × 10^7 (fMet-Leu-[^{3}H]Phe) or 1 × 10^8 cells/ml (fNle-Leu-[^{3}H]Phe). Dilutions of tritiated peptide from 10^{-10} to 2 × 10^{-7} M were prepared in the same protein-containing buffer as the cells. Duplicate reaction mixtures for the binding assay were set up at 4°C by layering 50 μl of the various dilutions of tritiated peptide over 500 μl Versilube F50 silicone oil (General Electric, Silicone Products Division, Waterford, NY) in a microfuge tube. The binding reaction was started by the addition of 50 μl of the appropriate concentration of cells and stopped after 15 min at 4°C by centrifugation in a microcentrifuge (model 235 A; Fisher Scientific Co., Pittsburgh, PA). Nonspecific binding was measured in the presence of a final concentration of 10^{-5} M nonradioactive peptide. The free peptide and silicone oil were aspirated from each reaction tube, and the tip of the tube that contained the cells was excised. The cell pellet was dissolved in 200 μl of 88% formic acid, and the cells were counted in Rackbeta scintillation counter (model 1217; LKB Instruments, Gaithersburg, MD).

The binding data were analyzed using a version of the LIGAND computer program (16) adapted for use on the Apple II computer by Dr. M. H. Teicher (Harvard Medical School, Cambridge, MA). The program was supplied by the Biomedical Computing Technology Information Center (Vanderbilt Medical Center, Nashville, TN). To test the hypothesis that toxin treatment had no effect on the binding of the peptide, we analyzed each set of binding data individually, then the combined data from control and toxin-treated cells in each experiment. The results were considered not to contradict the hypothesis if the residual variance between the experimental data and the fitted curves showed no significant increase in a partial F test as a result of applying the constraints that the control and toxin-treated cells should exhibit equal binding capacities and affinities. Computer estimates of nonspecific binding were used throughout these analyses; however, these estimates did not differ significantly from the experimental values of nonspecific binding.

Measurement of cAMP: The unstimulated and stimulated levels of intracellular cAMP in control and pertussis toxin–treated cells were measured as described previously (17). Briefly, 1-ml samples of washed cells (2 × 10^7 cells/ml) suspended in Hanks' buffer that contained 0.74 mM $MgCl_2$ were added to 12 × 75 mm Falcon disposable plastic tubes (Falcon Labware, Oxnard, CA). The reaction was started by the addition of 10^{-8} M fMet-Leu-Phe and stopped after 1 min by the addition of 15.0 μl of 30% ice cold trichloroacetic acid. The denatured suspensions were frozen-thawed once. Approximately 0.07 pmol of ^{3}[H]-cAMP was added to each sample for the determination of the recovery of cAMP. The samples were centrifuged at 2,400 *g* for 20 min, and 1 ml of the resulting supernatant was applied to a column (1.7 × 5 cm) of AG 50 W-X 8, H^+ form, analytical grade cation-exchange resin, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA) equilibrated with distilled water. The dried samples were reconstituted in 200 μl of 0.05 M sodium acetate buffer, pH 6.2 and to determine recovery, 50-μl aliquots were removed for liquid scintillation counting (Delta 300; Searle Analytic Inc). Recovery of cAMP was generally 85–95% and was determined for every sample. Aliquots of 50–75 μl were assayed for total cAMP content by radioimmunoassay, which was performed according to the protocol for nonacetylated samples supplied in a double antibody precipitation kit (New England Nuclear).

RESULTS

Inhibition by Pertussis Toxin of Granule Enzyme Secretion by Chemotactic Factors

The action of pertussis toxin shows a latent period. Incubation of rabbit neutrophils with 10–1,000 ng/ml pertussis toxin for 20 min at 37°C had no discernible effect on the ability of fMet-Leu-Phe to induce secretion of lysozyme or β-glucosaminidase (results not shown), whereas cells incubated longer were distinctly inhibited. Neutrophils pretreated with 10–1,000 ng/ml toxin for 45 min showed appreciable but submaximal inhibition of granule enzyme secretion (Fig. 1); at 60 min, the inhibition was indistinguishable from that obtained after a 90-min incubation (Fig. 1). As seen in Fig. 1, toxin treatment lowered the extent of release at every concentration of peptide tested. In the experiment pictured in Fig. 1, the inhibition was essentially complete in cells incubated with 200–1,000 ng/ml for 60 or 90 min. However, this varied somewhat among the four additional experiments performed; in some preparations of cells, inhibition was much more complete at 100 ng/ml than that shown in Fig. 1, whereas other cells were somewhat less sensitive to the action of the toxin. However, in all experiments, 200 ng/ml invariably gave maximal inhibition after a 60-min incubation. The results with β-glucosaminidase paralleled those with lysozyme, and there was no increase in lactic dehydrogenase leakage under any conditions of toxin treatment.

As is evident from Fig. 2, pretreatment of neutrophils at 37°C for 60 min with 500 ng/ml toxin caused complete inhibition of granule enzyme secretion induced by C5a or LTB_4. Despite appearances (Fig. 2), when the corresponding curves were analyzed as described for the binding studies, the lower concentrations of LTB_4 and C5a showed no significant differences in their ability to release enzyme from toxin-treated and untreated cells. The same pattern as in Fig. 2 was seen in one additional experiment.

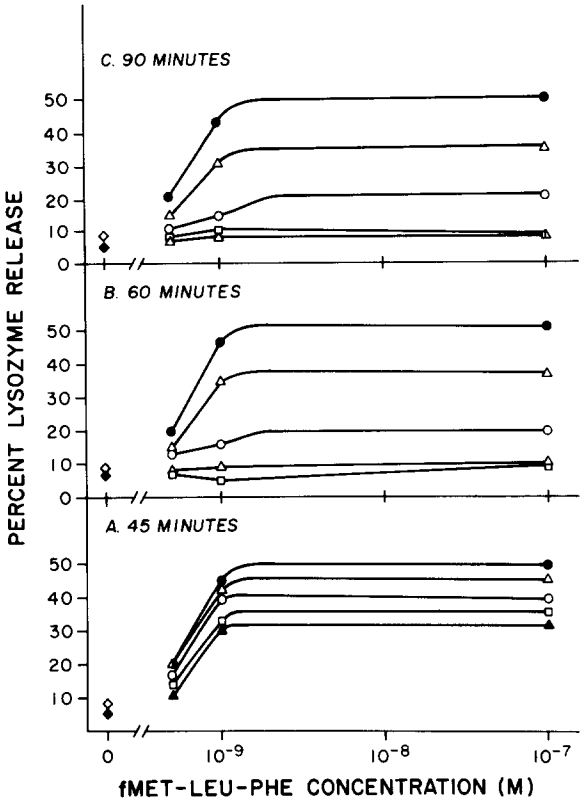

FIGURE 1 The effect of the treatment at 37°C of rabbit peritoneal neutrophils with varying concentrations of pertussis toxin for 45 min (*A*), 60 min (*B*), and 90 min (C) on the ability of the cells to secrete lysozyme in response to fMet-Leu-Phe. ●, no toxin; △, 10 ng/ml toxin; ○, 100 ng/ml toxin; □, 200 ng/ml toxin; ▲, 1,000 ng/ml toxin; ◇, cytochalasin B; ◆, blank.

Toxin Effect on Enzyme Release by PMA and A23187

As Fig. 3 demonstrates, incubation of rabbit neutrophils with 500 ng/ml toxin for 60 min at 37°C had no effect on lysozyme release induced by PMA. In the same experiment, toxin treatment completely abolished the secretagogue activity of fMet-Leu-Phe plus cytochalasin B. Chemotactic factors require cytochalasin B to release specific and azurophil granule contents from neutrophils in suspension, and cytochalasin B enhances release by A23187 (18). PMA does not require cytochalasin B to induce release of specific granule constituents but does need it to induce the secretion of azurophil granule enzymes (19). In the experiment pictured in Fig. 3 and in an additional experiment, cytochalasin B was not added to the PMA. In two other experiments not shown here, there was no inhibition of lysozyme or β-glucosaminidase secretion from toxin-treated neutrophils in the presence of 5 μg/ml cytochalasin B and PMA. Thus, neither the presence nor absence of cytochalasin B affects the inability of pertussis toxin to inhibit enzyme release by PMA.

The calcium ionophore A23187 also induces granule enzyme secretion from rabbit neutrophils (18). Incubation of rabbit neutrophils with 500 ng/ml toxin for 60 min at 37°C had no reproducible effect on either lysozyme release induced by A23187 (Fig. 4) or on glucosaminidase secretion (not shown). When the two curves of Fig. 2 were analyzed for equivalence essentially as described for the binding studies, the ED_{50} for the toxin-treated cells was 0.83×10^{-6} M, whereas

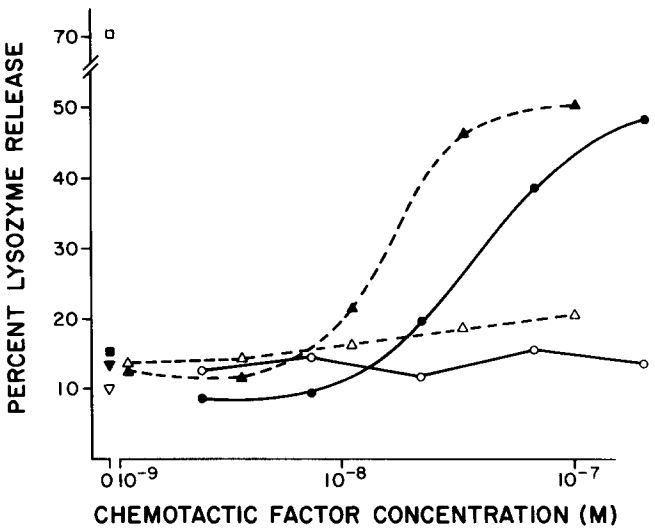

FIGURE 2 The effect of treating rabbit peritoneal neutrophils for 60 min at 37°C with 500 ng/ml of pertussis toxin on lysozyme secretion induced by LTB_4 and C5a. ●, LTB_4; ○, LTB_4 + 500 ng/ml toxin; ▲, C5a; △, C5a + 500 ng/ml toxin; □, fMLP (10^{-7} M); ■, fMLP (10^{-7} M) + 500 ng/ml toxin; ◇, blank; ◆, blank + cytochalasin B.

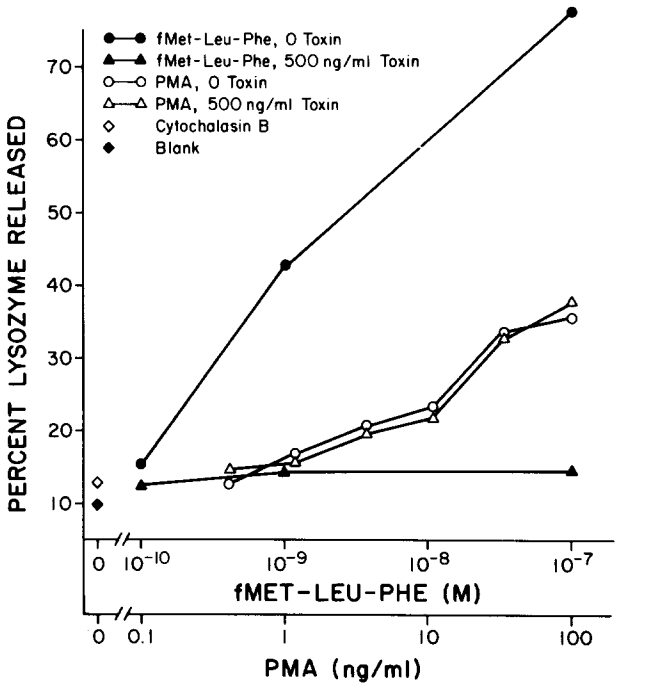

FIGURE 3 The lack of effect of treating rabbit peritoneal neutrophils for 60 min at 37°C with 500 ng/ml of pertussis toxin on lysozyme secretion induced by PMA. The secretion was measured after a 25-min incubation at 37°C of neutrophils with PMA.

that for the untreated control was 1.2×10^{-6} M, a small but statistically significant difference ($P < 0.02$). However, a second experiment done the same way did not confirm this result—the ED_{50} for the toxin-treated neutrophils was 4×10^{-7} M, and that for the untreated control was 2.6×10^{-7} ($P > 0.1$).

Toxin Inhibition of Chemotaxis

Fig. 5 shows that the pretreatment of neutrophils with the toxin also inhibited their chemotactic responsiveness to fMet-

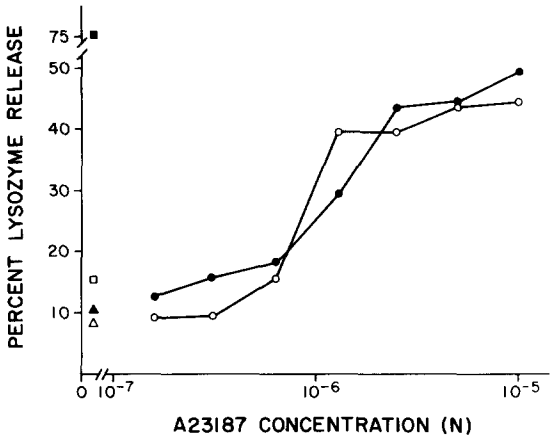

FIGURE 4 The effect of treating rabbit peritoneal neutrophils for 60 min at 37°C with 500 ng/ml of pertussis toxin on lysozyme secretion induced by A23187. ●, A23187; ○, A23187 + 500 ng/ml toxin; ■, fMLP (10^{-7} M); □, fMLP, (10^{-7} M) + 500 ng/ml toxin; △, blank; ▲, cytochalasin B.

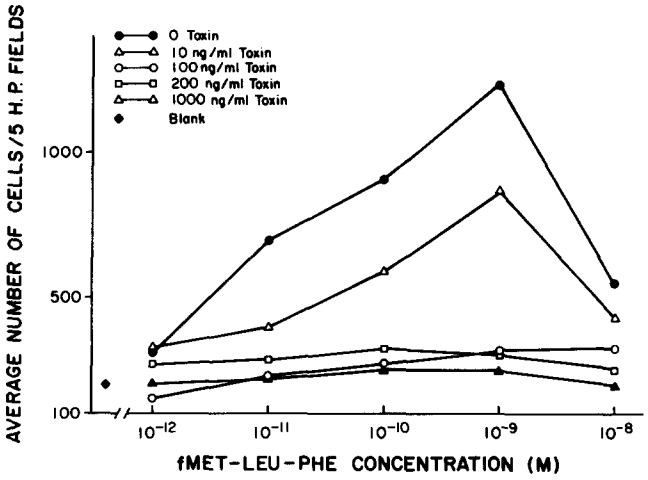

FIGURE 5 The inhibition of chemotaxis induced by treating rabbit peritoneal neutrophils with 500 ng/ml pertussis toxin for 60 min at 37°C.

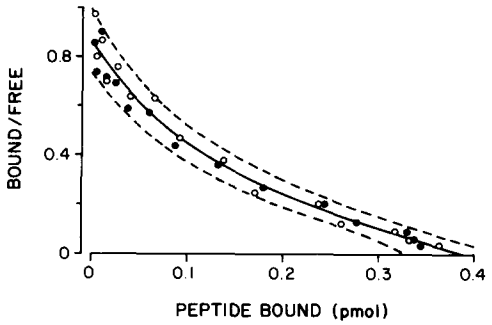

FIGURE 6 Scatchard plot of the binding of fNle-Leu-[^{3}H]Phe to (●) control rabbit peritoneal neutrophils and (○) neutrophils pretreated with 500 ng/ml pertussis toxin for 60 min at 37°C. The solid line represents the optimal curve fitted by the LIGAND computer program to the combined data for control and toxin-treated cells subject to the constraints that the two groups of cells have equal binding capacities and affinities. The broken lines represent approximate 95% confidence limits for data points about this fitted curve.

Leu-Phe. The sensitivity of the chemotactic reactivity to toxin treatment was at least as great as that of the granule enzyme secretory response and may be somewhat greater. The same results were seen in a duplicate experiment. Interestingly, there appeared to be no effect of toxin treatment on the penetration of the filter by the cells in the unstimulated buffer control.

Effect of Pertussis Toxin on Formyl Peptide Binding

Three studies of fNle-Leu-[^{3}H]Phe–specific binding were performed to assess the effect of the pretreatment of rabbit neutrophils with 500 ng/ml pertussis toxin at 37°C for 60 min. Scatchard plots of formyl peptide binding in one typical study are shown in Fig. 6. The Scatchard plots were invariably curved for both control and toxin-treated cells. Analysis by the LIGAND computer program indicated that the data were consistent with the binding of fNle-Leu-[^{3}H]Phe to two independent sets of binding sites: the equilibrium dissociation constants (K_d) for these sites averaged 1.0×10^{-9} and 1.2×10^{-8} M and 8% of the sites, on average, were of the higher affinity. In the study illustrated (Fig. 6), the fit to the complete experimental data for both control and toxin-treated cells was not significantly worsened ($F = 1.29$ with 4 and 20 degrees of freedom; $P > 0.2$) under the constraints that the binding capacities and affinities were unaffected by toxin treatment. Moreover, the experimental data points for both control and toxin-treated cells all lay within the 95% confidence limits around the constrained composite curve. Data from the other two studies with fNle-Leu-[^{3}H]Phe showed the same pattern. Toxin treatment again did not significantly affect the binding of formyl peptide to either binding site ($F = 0.19$ and 0.76; $P > 0.5$ in each case).

Four studies were performed with fMet-Leu-[^{3}H]Phe (data not shown). The curvature of the Scatchard plots was less definitive with this formyl peptide, and the K_d values for the two sites (6.8×10^{-10} and 4.1×10^{-9} M) were lower than with fNle-Leu-[^{3}H]Phe. In two of the studies, toxin treatment had no significant effect on the binding of this formyl peptide ($F = 2.60$ and 0.97; $P > 0.05$ and $P > 0.5$, respectively); in the third and fourth, however, a small increase in one and decrease in the other in the overall binding capacity appeared to occur after toxin treatment.

Effect of Pertussis Toxin on the Basal and fMet-Leu-Phe–stimulated Levels of cAMP

We also examined the effect of pretreatment of rabbit neutrophils with toxin on the basal and fMet-Leu-Phe–stimulated rise in intracellular level of cAMP. The results summarized in Table I clearly show that the addition of toxin does not significantly affect the basal or the stimulated cAMP level.

DISCUSSION

The ability of pertussis toxin under the appropriate conditions to completely inhibit chemotaxis and granule enzyme secretion induced by the chemotactic factors, fMet-Leu-Phe, C5a, and LTB_4 is strong evidence for the presence of a guanine nucleotide binding regulatory protein in the neutrophil identical with or analogous to N_i or to transducin. In an abstract that appeared as this work was being prepared for publication, Goldman et al. (20) reached a similar conclusion when they

TABLE I

Effect of Pretreatment with Pertussis Toxin on the Basal and fMet-Leu-Phe–stimulated cAMP in Rabbit Neutrophils

		Intracellular level of cAMP	
	Time	Basal	fMet-Leu-Phe
	min	*pmol/10^7 cells*	
Control cells	0	6.9 ± 0.4	9.5 ± 0.3
	30	6.4 ± 0.25	10.5 ± 0.25
	60	7.0 ± 0.3	9.0 ± 0.15
Toxin-treated cells	0	6.1 ± 0.2	9.0 ± 0.2
(500 ng/ml)	30	6.3 ± 0.3	9.9 ± 0.3
	60	6.5 ± 0.25	9.5 ± 0.25

Time is in minutes at 37°C with or without the addition of the toxin. The cells were treated with 10^{-8} M fMet-Leu-Phe for 1 min before the reaction was stopped. The values represent the mean ± SEM of at least three determinations.

also found that pertussis toxin inhibits the activation of human neutrophils by chemotactic factors. More direct evidence for this conclusion are the unpublished findings of F. Okajima and M. Ui (referred to in reference 21) and C. K. Huang of this institution, that pertussis toxin induces ADP-ribosylation of a 41-kD membrane protein from guinea pig and rabbit neutrophils, respectively. The inhibition of neutrophil activity by pertussis toxin is also strong evidence that the process or processes that this N protein regulates plays a necessary role in neutrophil function. The inhibition of both enzyme secretion and chemotaxis by pertussis toxin is concentration dependent and, most tellingly, its action requires a latent period. Presumably, as in all other cells where it has been tested, the latent period is required for the pertussis toxin to bind to the cell and be internalized to exert its function. The neutrophil possesses a protein identical with or similar to N_s (7). Cholera toxin which acts through N_s to raise cAMP levels inhibits stimulated neutrophil function (8, 9). However, no matter what concentration of toxin or time of preincubation is used, the inhibition is never more than 40–50 %. This suggests that unlike the process regulated by the N protein susceptible to pertussis toxin, the neutrophil process or processes affected by N_s may not be absolutely required for cell function or that there are pathways involved in the cell functions that are independent of the latter.

One possible mechanism of the inhibition is that the toxin treatment affects chemotactic receptor binding. Pertussis toxin treatment decreases the affinity of the D_2 receptor for dopamine (22) and the α_2-adrenergic, the cholinergic, and opiate receptors for their respective ligands (23). Garcia-Saenz et al. (24) have reported that pertussis toxin treatment induces a decreased formation of the high-affinity state for α_2-adrenergic agonists. Neutrophils contain high- and low-affinity receptors for the formylpeptides (25, 26), and guanine nucleotides have been reported to transform the high-affinity state of the formylpeptide receptor of neutrophils and macrophages into a lower affinity state (10). However, pertussis toxin treatment of neutrophils sufficient to completely inhibit the secretagogue action of fMet-Leu-Phe has no detectable effect on either the affinity or number of high- or low-affinity formylpeptide binding sites. This indicates that toxin treatment does not act on receptor binding but at some process or processes after receptor-chemotactic factor interaction. It is unknown whether the lack of effect of toxin treatment on receptor binding is because the putative N protein does not mediate changes in receptor affinity or whether, with the experimental conditions used, guanine nucleotide regulation of the binding site is nonexistent or not prominent.

In many cells, N_i is coupled to adenylate cyclase, and in these cells, treatment with pertussis toxin prevents the inhibition of adenylate cyclase which results from receptor-agonist interaction (3, 4). This does not appear to be true of chemotactic factor-receptor interaction in neutrophils. Treatment of neutrophils with toxin under conditions that completely inhibit the ability of fMet-Leu-Phe to induce granule enzyme secretion has no significant effect on the level of cAMP in the same cells. fMet-Leu-Phe induces a rapid, transient increase in neutrophil intracellular cAMP (17, 27) which, however, is not required for the activation involved in granule secretion or the generation of O_2^- (28). Toxin treatment of the neutrophils causes no change in the transient rise induced by fMet-Leu-Phe. Although it is not known whether the receptor-mediated rise of cAMP occurs through the activation of adenylate cyclase, this finding does emphasize that the effect of pertussis toxin is not mediated through an increase of cAMP. This is again in contrast to the action of cholera toxin, in which the inhibitory action has been associated with such a rise (8, 9). Nakamura and Ui (29) have reported that cAMP does not appear to play a role in the inhibition of stimulated histamine release in rat mast cells treated with pertussis toxin. These findings suggest that in both the mast cell and in the neutrophil, the N protein acted upon by pertussis toxin may not be coupled to adenylate cyclase.

Our ignorance as to the nature of the putative GTP-binding regulatory protein that is the presumed target of the pertussis toxin extends to the general nature of the process that is inhibited. There are at present two possibilities. The first is that the toxin, in reacting with its presumed neutrophil N protein, interferes with the inhibition by that activated N protein of some receptor-mediated process; this would be analogous to the manner in which the toxin in other cells interacts with N_i to prevent its inhibition of adenylate cyclase. The second possibility is that the toxin, in interacting with N protein, prevents its activation of some necessary process; the analogy here is the way pertussis toxin prevents retinal transducin from activating the cGMP phosphodiesterase (1).

This work, although throwing no light on the function of the target of toxin action other than providing some examples of what it is not, clearly pinpoints the area in the process of neutrophil activation where this function is exerted. At present, most investigators believe that chemotactic factors such as fMet-Leu-Phe, C5a, LTB_4, etc., activate neutrophils through, among other things, the combined action of a rise in cytosolic Ca^{2+} with its resultant reactions, and the activation of protein kinase C and the reactions that ensue from this. The lack of effect of pertussis toxin on the granule enzyme release induced by PMA indicates that the action of pertussis toxin does not involve the sequence of reactions initiated by the activation of protein kinase C.

Treatment of the cell with toxin also does not affect granule enzyme release initiated by the influx of Ca^{2+} caused by the Ca^{2+} ionophore, A23187. This is consistent with the conclusion that the final locus of toxin action is at one or more of the reactions that occur between chemotactic receptor stimulation and the resultant rise in intracellular Ca^{2+}. This conclusion is also fully supported by our recent finding that the rise in intracellular Ca^{2+} induced by fMet-Leu-Phe or LTB_4 is greatly reduced or abolished in pertussis toxin–treated neutrophils (30). Which reaction or reactions is the ultimate

target of toxin action in the sequence that precedes the rise in intracellular Ca^{2+} is presently under study. In the course of this study, we have recently found (31) that the addition of pertussis toxin to rabbit neutrophils inhibits the fMet-Leu-Phe–induced rises in Na^+ influx, intracellular pH, and the stimulated changes in polyphosphoinositides and the phosphorylation of a 46-kD protein. Unlike the effect of fMet-Leu-Phe, the increases in Na^+ influx and intracellular pH produced by PMA are not inhibited by the toxin. Also in accordance with the present results is the recent observation that exposure of guinea pig neutrophils to pertussis toxin results in a marked inhibition of the arachidonic acid release from these cells that occurs after chemotactic factor stimulation (referred to in references 21 and 29; see also references 32 and 33). After this manuscript was submitted, Okajima and Ui (32) and Bokoch and Gilman (33) published conclusions similar to those researched here.

The work was partially supported by National Institutes of Health grants AI 09648 to Dr. Becker, AI 13734 to Dr. Sha'afi, and AM 31000 to Dr. Naccache.

Received for publication 16 November 1984, and in revised form 11 January 1985.

REFERENCES

1. Houslay, M. D. 1984. A family of nucleotide regulatory proteins. *Trends Biochem. Sci.* 9:139–40.
2. Moss, J., and M. Vaughan. 1979. Activation of adenylate cyclase by choleragen. *Annu. Rev. Biochem.* 48:581–600.
3. Ui, M. 1984. Islet-activating protein, pertussis toxin: a probe for functions of inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol. Sci.* 5:277–279.
4. Katada, T., and M. Ui. 1982. Direct modification of the membrane adenylate cyclase system by islet activating protein due to ADP-ribosylation of a memebrane protein. *Proc. Natl. Acad. Sci. USA.* 79:3129–3133.
5. Sewell, W. A., J. J. Munoz, and M. A. Vadas. 1983. Enhancement of the intensity, duration and passive transfer of delayed hypersensitivity by pertussigen in mice. *J. Exp. Med.* 157:2087–2096.
6. Arai, H., and J. J. Munoz. 1981. Crystallization of pertussingen from Bordetella pertussis. *Infect. Immun.* 31:495–499.
7. Lad, P. M., M. M. Glovsky, P. A. Smiley, M. Klempner, D. M. Reissinger, and J. M. Richards. 1984. The β-adrenergic receptor in the human neutrophil plasma membrane: receptor cyclase uncoupling is associated with amplified GTP activation. *J. Immunol.* 132:1466–1471.
8. Rivkin, I., J. Rosenblatt, and E. L. Becker. 1975. The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. The inhibition of neutrophil movement and elevation of cyclic AMP levels by catecholamines, prostaglandins, theophylline and cholera toxin. *J. Immunol.* 115:1114–1134.
9. Hill, H. R., R. D. Estenson, P. G. Quie, N. A. Hogan, and N. D. Goldberg. 1975. Modulation of human neutrophil chemotactic responses by cyclic 3′, 5′ guanosine monophosphate and cyclic 3′, 5′ adenosine monophosphate. *Metabolism.* 24:447–456.
10. Snyderman, R. 1984. Regulatory mechanisms of a chemoattractant receptor on leukocytes. *Fed. Proc.* 43:2743–2748.
11. Hyslop, P. A., Z. G. Oades, A. J. Jesaitis, R. G. Painter, C. G. Cochrane, and L. A. Sklar. 1984. Evidence for *N*-formyl chemotactic peptide-stimulated GTPase activity in human neutrophil homogenates. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 166:165–169.
12. Showell, J. H., R. J. Freer, S. H. Zigmond, E. Schiffmann, S. Aswanikumar, B. A. Corcoran, and E. L. Becker. 1976. The structure activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.* 143:1154–1169.
13. Fantone, J. C., R. M. Senior, D. L. Kreutzer, M. Jones, and P. A. Ward. 1979. Biochemical quantitation of the chemotactic inactivator activity in human serum. *J. Lab. Clin. Med.* 93:17–24.
14. Swanson, M. J. 1977. A simple multiple chamber apparatus for measuring chemotaxis of polymorphonuclear leukocytes utilizing centrifugation of the chamber before incubation. *J. Immunol. Methods.* 16:385–390.
15. Mackin, W. M., C.-K. Huang, B. J. Bormann, and E. L. Becker. 1983. A simple and rapid assay for measuring radiolabeled ligand binding to purified plasma membranes. *Anal. Biochem.* 131:430–437.
16. Munson, P. J., and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* 107:220–239.
17. Jackowski, S., and R. I. Sha'afi. 1979. Response of adenosine cyclic 3′,5′ monophosphate level in rabbit neutrophils to the chemotactic peptide, formylmethionyl-leucyl-phenylalanine. *Mol. Pharmacol.* 16:473–478.
18. Showell, H. J., P. H. Naccache, R. I. Sha'afi, and E. L. Becker. 1977. The effects of extracellular K^+, Na^+ and Ca^{2+} on lysosomal enzyme secretion from polymorphonuclear leukocytes. *J. Immunol.* 119:804–811.
19. Ringel, E. W., N. A. Soter, and K. F. Austen. 1984. Localization of histaminase to the specific granule of the human neutrophil. *Immunology.* 52:649–658.
20. Goldman, D. W., C. A. Gifford, H. R. Bourne, and E. J. Goetzl. 1984. Pertussis toxin (PT) inhibits the activation of human neutrophils (Ns) to chemotactic factors (CFs). *J. Cell. Biol.* 99 (4, Pt. 2): 278*a*. (Abstr.)
21. Nakamura, T., and M. Ui. 1984. Islet-activating protein, pertussis toxin, inhibits Ca^{2+} induced and guanine nucleotide-dependent releases of histamine and arachidonic acid from rat mast cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 173:414–418.
22. Kuno, T., O. Shirakawa, and C. Tanaka. 1983. Selective decrease in the affinity of D_2 dopamine receptor for agonist induced by islet-activating protein, pertussis toxin, associated with ADP-ribosylation of the specific membrane protein of the bovine striatum. *Biochem. Biophys. Res. Commun.* 115:325–330.
23. Kurose, H., T. Katada, T. Amono and M. Ui 1983. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via adrenergic cholinergic and opiate receptors in neuroblastoma α glioma hybrid cells. *J. Biol. Chem.* 258:4870–4875.
24. Garcia-Saenz, J. A., J. L. Boyer, T. Michel, D. Sawyer, G. L. Stiles, H. Dohlman, and R. J. Lefkowitz. 1984. Effect of pertussis toxin on α_2 adrenoreceptors: decreased formation of the high affinity state for agonists. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 172:95–98.
25. Mackin, W. M., C. K. Huang, and E. L. Becker. 1982. The formylpeptide chemotactic receptor on rabbit peritoneal neutrophils. 1. Evidence for two binding sites with different affinities. *J. Immunol.* 129:1608–1611.
26. Koo, C., R. J. Lefkowitz, and R. Snyderman. 1982. The oligopeptide chemotactic factor receptor on human polymorphonuclear leukocyte membranes exist in two affinity states. *Biochem. Biophys. Res. Commun.* 106:442–449.
27. Keller, H. U., G. Gerisch, and J. H. Wissler. 1979. A transient rise in cyclic AMP levels following chemotactic stimulation of neutrophil granulocytes. *Cell Biol. Int. Rep.* 3:759–765.
28. Simchowitz, L., I. Spilberg, and J. P. Atkinson. 1983. Evidence that the functional responses of human neutrophils occur independently of transient elevations in cyclic AMP levels. *J. Cyclic Nucleotide Protein Phosphorylation Res.* 9:35–47.
29. Nakamura, T., and M. Ui. 1983. Suppression of passive cutaneous anaphylaxis by pertussis toxin, an islet activating protein, as a result of inhibition of histamine release from mast cells. *Biochem. Pharmacol.* 32:3435–3441.
30. Molski, T. F. P., P. H. Naccache, M. L. Marsh, J.Kermode, E. L. Becker, and R. I. Sha'afi. 1984. Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemotactic factors in rabbit neutrophils: possible role of the "G proteins" in calcium mobilization. *Biochem. Biophys. Res. Commun.* 124:644–650.
31. Volpi, M., P. H. Naccache, T. F. P. Molski, J. Shefcyk, C.-K. Huang, M. L. Marsh, J. Munoz, E. L. Becker, and R. I. Sha'afi. 1985. Pertussis toxin inhibits the formylmethionyl-leucyl-phenylalanine but not the phorbol ester stimulated changes in ion fluxes, protein phosphorylation and phospholipid metabolism in rabbit neutrophils: role of the "G-proteins" in excitation response coupling. *Proc. Natl. Acad. Sci. USA.* In press.
32. Okajima F., and M. Ui. 1984. ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. A possible role of the toxin substrate in Ca^{2+} mobilizing biosignalling. *J. Biol. Chem.* 259:13863–13871.
33. Bokoch, G. M., and A. G. Gilman. 1984. Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell.* 39:301–308.