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Bordetella pertussis Adenylate Cyclase: Effects of Affinity-Purified Adenylate Cyclase on Human Polymorphonuclear Leukocyte Functions

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Affinity-purified adenylate cyclase (AC) of *Bordetella pertussis*, free of contaminating pertussis toxin, was demonstrated to have biological effects on human polymorphonuclear leukocytes (PMN). AC at doses of 25 and 50 μg/ml increased intracellular cAMP levels in the phagocytes 7.6- to 23.5-fold, respectively, above basal levels. AC inhibited PMN chemiluminescence, chemotaxis, and superoxide production in a dose-dependent manner. The 50% inhibitory dose for chemotaxis and chemiluminescence was 36.5 μg/ml; for superoxide generation it was 71.0 μg/ml. Although these PMN metabolic functions were impaired, no effect on phagocytic activity was observed.

Previous studies on the effects of *Bordetella pertussis* adenylate cyclase (AC) on eucaryotic cell functions have been done with crude urea extracts of bacterial cells (8, 15, 16, 29). These preparations contain a complex mixture of proteins of *B. pertussis*, including pertussis toxin (PT), filamentous hemagglutinin, and other surface proteins as well as AC (12). Thus, the observed inhibitory effects reported to be due to AC may be caused by other exoproducts in the crude preparations. Recent reports have shown that PT inhibits various functions of phagocytic cells (1, 14, 21, 23). For any critical studies on the role of AC in *B. pertussis* pathogenesis, purified preparations of AC are required that have both enzymatic and biological activities. Attempts have been made to purify AC, but only the enzymatic activity was recovered (16).

Shattuck et al. (27, 28) reported the partial purification of *B. pertussis* AC, which has enzymatic activity that is activated by calmodulin and which induces increases in cAMP in erythrocytes and N1E-115 neuroblastoma cells. Low yields of AC were recovered, i.e., 6 μg from 18 liters of culture supernatant (27). Recently, Kessin and Franke (18) partially purified from culture supernatant AC which has enzymatic activity stimulated by calmodulin (18). It was not reported in these studies whether the isolated AC has inhibitory biological effects on phagocytic cells. In a previous paper, we reported the isolation and purification of AC by calmodulin-affinity chromatography (12). Affinity-purified AC has enzymatic activity which is stimulated by calmodulin; it was found to be free of PT. In this paper, we report the biological effects of affinity-purified AC on human polymorphonuclear leukocyte (PMN) functions.

MATERIALS AND METHODS

Affinity purification of AC. *B. pertussis* AC from crude urea extracts was isolated and affinity purified by calmodulin-Sepharose 4B chromatography as previously described (12). AC preparations were stored at −70°C.

Human PMN preparations. PMN were isolated from venous blood treated with heparin. For the chemiluminescence (CL) and chemotaxis (CT) studies, isolation was performed by hydroxyethyl starch sedimentation and centrifugation through Ficoll-Hypaque as described by Boyum (6). PMN were then washed and suspended in gel-Hanks balanced salt solution at 2×10^6 PMN per ml. For superoxide studies, heparinized blood was separated by the one-step Ficoll-Hypaque method of Ferrante and Thong (11). The PMN were washed in Dulbecco phosphate-buffered saline supplemented with 0.1% bovine serum albumin and 3 mg of glucose per ml (pH 7.4). Contaminating erythrocytes were eliminated by hypotonic lysis. PMN were resuspended in Dulbecco phosphate-buffered saline at a concentration of 2×10^6 PMN per ml.

PMN were incubated with various concentrations of AC for 1 h in polypropylene tubes at 37°C with the use of a Labquake tube rotator (LabIndustries, Berkeley, Calif.). After preincubation, PMN were washed or used directly in various assays. Viability before and after cyclase treatment was monitored by trypan blue exclusions. Control PMN viability was observed to be 99.7%, and AC-treated cell viability was 99.3, 99.3, 98.0, and 91.3% at doses of 5, 25, 50, and 100 μg/ml, respectively.

PMN intracellular cAMP levels. PMN were incubated with crude or purified AC or with 0.1 mM forskolin (Calbiochem-Behring, La Jolla, Calif.) for 1 h at 37°C. Cells were recovered by centrifugation and extracted by the addition of 0.5 ml of 50 mM sodium acetate buffer (pH 4.2) containing 0.1 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co., St. Louis, Mo.) and sonicated for 30 s on ice. Samples were boiled for 5 min, and cellular debris was removed by centrifugation. The cAMP concentration of the supernatants was determined by using an Amersham cAMP radioimmunoassay kit (Amersham Corp., Arlington Heights, Ill.).

CL assay. CL assays were done by the method of Lindberg et al. (20). Briefly, 5×10^5 PMN in a 250-μl volume was added to dark-adapted glass scintillation vials containing 1 ml of luminol (Sigma)-saturated fetal calf serum (~0.2 mg/ml) and 4.5 ml of gel-Hanks balanced salt solution. CL was initiated by the addition of 3×10^7 heat-killed *Staphylococcus aureus* which had been opsonized with fresh serum for 30 min at 37°C (bacteria to PMN ratio of 60:1). Vials were counted at room temperature by a Beckman LS-250 scintillation counter (Beckman Instruments, Inc., Fullerton,

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TABLE 1. Ability of crude and affinity-purified AC to induce intracellular accumulation of cAMP in human PMN

PMN treatment[a]	Concn (μg/ml)	cAMP (pmol/10^7 PMN) ± SD[b]
None		2.0 ± 0.3
Forskolin	0.1 (mM)	24.0 ± 1.0
Crude AC	300	53.4 ± 1.3
	500	135.2 ± 12.1
Affinity-purified AC	25	15.1 ± 4.0
	50	47.0 ± 9.5

[a] PMN were preincubated with crude or purified AC or forskolin for 1 h at 37°C, washed, and assayed for cAMP.
[b] Results are the means of three duplicate experiments.

Calif.) by using the ^3H window. Vials were counted for 1 min, and the counting cycle was repeated for 2 to 3 h without mixing of vials. Results were expressed as counts per minute (cpm experimental minus background before the addition of *S. aureus*) and presented as the percentage of maximum CL intensity (peak response) of the control.

Phagocytosis. Phagocytosis activity of control and AC-treated PMN was monitored during the CL assays. Samples were set up in duplicate, mixed, and allowed to sit at room temperature for 1 h as in the CL assay described above. Contents of the vials were poured into 15-ml conical tubes and centrifuged gently (50 × *g* for 5 min) to recover PMN. The cells were washed two times in gel-Hanks balanced salt solution, and the cell pellet was suspended in 3 drops of autologous serum. Smears were made on glass slides by use of a Larc blood spinner (Corning Glass Works, Corning, N.Y.). Slides were spun for 2 to 3 s, air dried, fixed in absolute methanol, and stained with Giemsa solution for 30 min. Slides were washed with water, air dried, and observed microscopically for phagocytosis. The percentage of PMN phagocytosing *S. aureus* (percent phagocytosis) and the ratio of the number of bacteria phagocytosed to PMN was determined from counting 100 cells each of control and AC-treated PMN at the various concentrations tested.

CT assay. CT of normal and AC-treated PMN was monitored by the PMN-migration-under-agarose technique of Glasser and Fiederlein (13). Four wells 2.5 mm in diameter were punched into agarose plates 2.5 mm from a well in the center of the plate. Control or AC-treated PMN were placed in the outside wells (10^5 PMN in a 5-μl volume), and fresh serum was added to the center as the chemotactic agent (fresh serum reacts with agarose to produce chemotactic complement components). Plates were incubated at 37°C in a humidified CO_2 incubator for 5 h. The plates were removed, inverted in a refrigerator for 1 to 2 h, fixed with absolute methanol followed by 10% buffered formalin. The agarose was removed, and PMN were stained with Giemsa. A scoring system combining both cell counts and the distance migrated was used to determine chemotactic activity (13). Results are given as percentage of the CT response of the control.

Superoxide generation. PMN superoxide generation that resulted from exposure to *S. aureus* was measured by the procedure of Curnutte and Babior (9). Briefly, in a final volume of 1.0 ml, 2 × 10^5 control or AC-treated PMN were mixed either with or without superoxide dismutase and with 2 × 10^7 heat-killed *S. aureus* which had been opsonized with 20% human serum for 30 min at 37°C. All mixtures also contained cytochrome C (80 μM) and 10% human serum. After 30 min of incubation at 37°C, the preparations were chilled in an ice bath, centrifuged for 30 min at 4°C, and additionally centrifuged for 3 min at 15,600 × *g* (Eppendorf Micro Centrifuge; Brinkman Instruments, Inc., Westbury, N.Y.) to clear the supernatants. Supernatants were then assayed for superoxide-dependent reduction of ferricytochrome C by optical density measurements at 550 nm.

Statistical analysis. The significance of differences between results obtained under different exposures of PMN to AC was calculated by the Student's *t* test or by the Wilcoxon signed-ranks test.

RESULTS

Ability of AC to induce increases in PMN intracellular cAMP levels. Experiments were done to study the ability of crude and affinity-purified AC to induce increases in intracellular cAMP levels in PMN. Control PMN intracellular cAMP level was 2.0 ± 0.3 pmol/10^7 cells (Table 1). Forskolin at a 0.1 mM concentration (positive control) induced a 12-fold increase in PMN intracellular cAMP. Crude AC at 300 and 500 μg/ml induced 26.7- and 67.6-fold increases, respectively, in intracellular cAMP. Affinity-purified AC was also observed to significantly increase intracellular cAMP levels in PMN to 15.1 ± 4.0 and 47.0 ± 9.5 pmol/10^7 cells at doses of 25 and 50 μg/ml, respectively. This was a 7.6- and a 23.5-fold increase, respectively. These results demonstrate that the affinity-purified AC, like crude AC, had the ability to induce increases in intracellular cAMP in human PMN.

Effects of AC on PMN CL response and phagocytosis. PMN preincubated for 1 h with various concentrations of affinity-purified AC were observed to inhibit neutrophil CL response to *S. aureus* in a dose-dependent manner (Fig. 1A). CL activity was inhibited to 88.9 ± 8.4, 60.4 ± 17.5, 36.0 ± 15.1, and 13.8 ± 8.5% of control levels at doses of 5, 25, 50, and 100 μg of AC per ml, respectively. The inhibition was highly

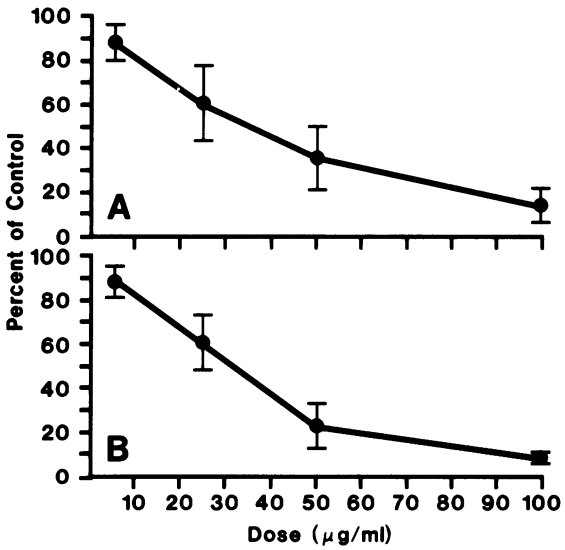

FIG. 1. Inhibition of human PMN CL and CT responses by *B. pertussis* affinity-purified AC. PMN (2 × 10^6/ml) were preincubated with various doses of AC and assayed for CL (A) or CT (B). The CL response is reported as the percentage of the control-level maximum CL intensity. Maximum control-level CL response of PMN was (4.3 × 10^5 cpm ± 4.5 × 10^4)/5 × 10^5 PMN. CT is reported as the percent control-level migration response. The control-level migration was 950 ± 195 (13). The differences between the CL and CT responses of the control and AC-treated PMN were significant ($P < 0.001$) at all doses tested. The data are the means ± SD values from eight (CL) or four (CT) duplicate experiments.

significant ($P < 0.001$) compared with control levels at all doses tested. Control PMN reached a maximum CL response after 60 min of counting, whereas AC-treated cell response was suppressed and reached a maximum by 80 min (Fig. 2). No difference was observed in CL inhibition when PMN were incubated with affinity-purified AC and either washed or used directly in assays. The dose of affinity-purified AC that caused 50% inhibition of CL response was 36.5 μg/ml.

Light microscopy studies were done to determine whether the decrease in CL of AC-treated PMN was due to direct inhibition of phagocytosis. Phagocytosis occurred at similar levels for control and AC-treated (100 μg/ml) PMN (Fig. 3).

At all doses, 100% of the PMN had phagocytosed *S. aureus*. The number of *S. aureus* phagocytosed by controls was 28.3 ± 6 per PMN, whereas AC-treated PMN phagocytosed 24.5 ± 4, 29.8 ± 5, 31.1 ± 8, and 30.8 ± 6 bacteria per PMN at doses of 5, 25, 50, and 100 μg/ml, respectively. There was no significant difference between numbers of bacteria phagocytosed by control or AC-treated PMN.

Inhibition of PMN CT response by affinity-purified AC. The chemotactic response of PMN was inhibited in a dose-dependent fashion (Fig. 1B). CT activity was inhibited to 88.9 ± 6.4, 58.5 ± 11.5, 23.1 ± 10.8, and 8.5 ± 1.5% of control levels at AC doses of 5, 25, 50, 100 μg/ml, respectively. Inhibition at all doses was highly significant compared with control levels ($P < 0.001$). The dose of affinity-purified AC that caused 50% inhibition of CT was 36.5 μg/ml. A representative experiment of migration under agarose which shows the differences in cell migration and distance migrated by normal and AC-treated PMN is presented in Fig. 4.

Inhibition of PMN superoxide generation by AC. Preincubation of PMN for 1 h at AC doses of 20, 50, or 100 μg/ml was observed to inhibit superoxide generation during phagocytosis of *S. aureus* (Fig. 5). Superoxide generation was inhibited to 98.6 ± 2.9, 63.8 ± 17.6, and 31.9 ± 12.8% of control levels at AC doses of 20, 50, and 100 μg/ml. The inhibition was significant ($P < 0.05$) compared with controls at all doses tested. The AC dose required for 50% inhibition of superoxide generation was 71.0 μg/ml.

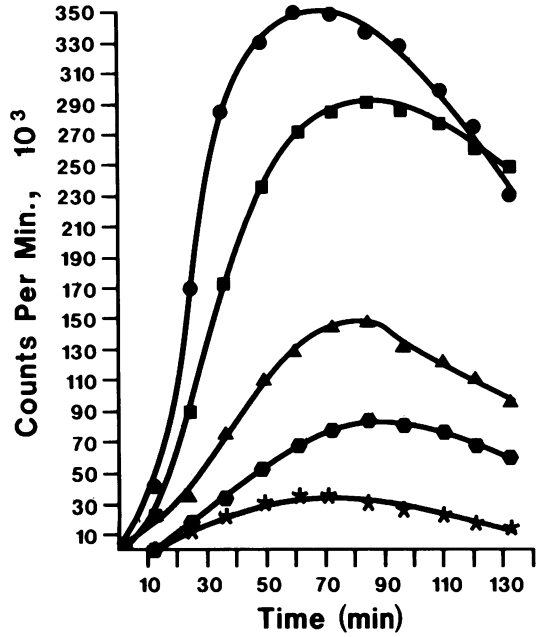

FIG. 2. Inhibition of human PMN CL response by *B. pertussis* affinity-purified AC. This is an example of a typical CL assay showing the response of control PMN (●) and PMN treated with 5 (■), 25 (▲), 50 (⬣), or 100 (*) μg of affinity-purified AC per ml. Inhibition at these doses was 17, 61, 76, and 89.4%, respectively.

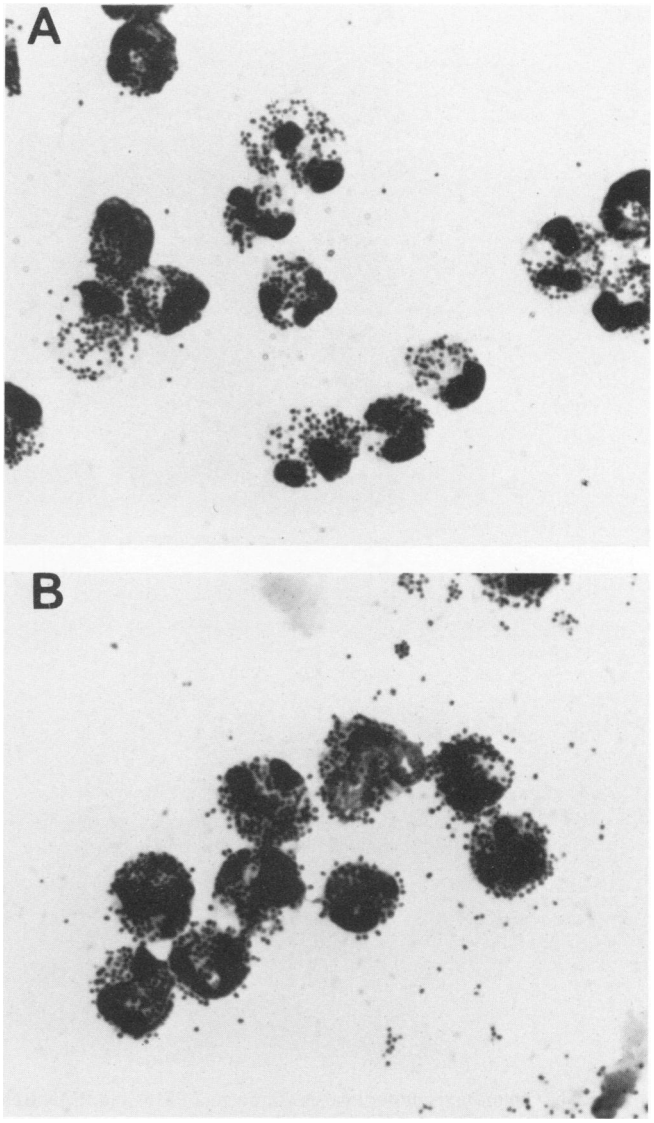

FIG. 3. Phagocytosis of heat-killed *S. aureus* by normal and AC-treated PMN. By light microscopy, normal PMN (A) and PMN preincubated with AC at 100 μg/ml (B) phagocytosed opsonized *S. aureus* to similar levels. Magnification, ×13,000.

Effect of PT on CL and CT responses of PMN. PMN were preincubated with PT, purified by the method of Sekura et al. (26), at various concentrations for 1 h, washed, and used in CL and CT experiments. PT at doses of 1, 2, 5, and 10 ng/ml had no significant inhibitory effect on neutrophil CL response (Table 2). Only at a dose of 100 ng/ml was significant inhibition observed. The CT response of PMN was not inhibited by PT at doses of 1 or 2 ng/ml, whereas inhibition occurred at doses of 5, 10, and 100 ng/ml (Table 2).

DISCUSSION

The existence of a *B. pertussis* AC was first reported by Wolff and Cook (33) in studies with whole-cell vaccine.

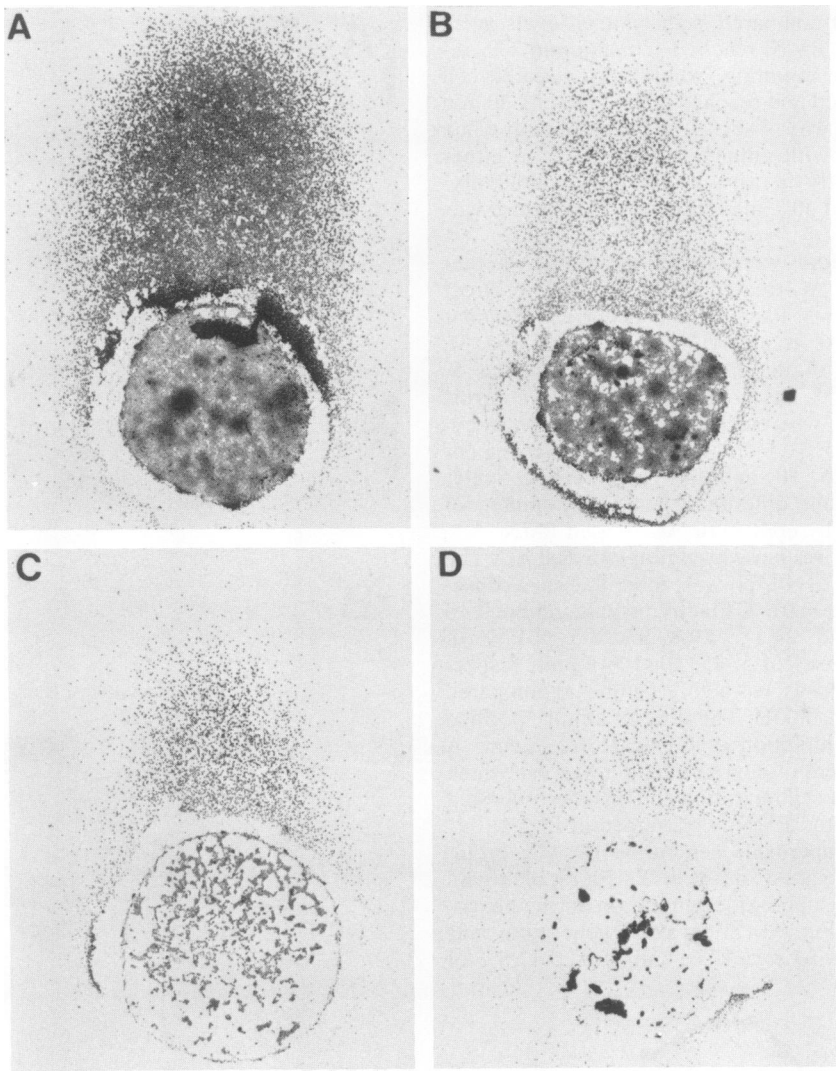

FIG. 4. Chemotaxis under agarose of normal PMN and PMN treated with affinity-purified AC. PMN were preincubated with (A) 0, (B) 25, (C) 50, or (D) 100 μg of AC per ml. The total number of AC-treated cells (B to D) migrating toward the chemoattractant and the distance migrated can be compared to results with normal PMN (A).

Hewlett and Wolff (16) demonstrated that AC accumulates to maximum levels in culture supernatant by 24 h. The majority of the AC activity was found to be associated extracytoplasmically with the bacterial cell surface (16). Studies by Utsumi et al. (29) showed that the AC can be recovered from *B. pertussis* cells by urea extraction and that the AC activity is quite stable under these conditions. The extract was found to inhibit human PMN functions, including chemotaxis and killing of *S. aureus*. The crude urea extract was found to induce accumulation of cAMP in various mammalian cell types, including PMN (8), lymphocytes (15), monocytes, CHO cells, mouse S49 lymphoma cells, and isolated rat pituitary cells (17). Confer and Eaton (8) hypothesize that *B. pertussis* AC enters phagocytic cells, is activated by calmodulin, and induces increased intracellular cAMP levels, which impairs PMN and macrophage bactericidal functions.

Although these reports are interesting, it cannot be conclusively stated that the inhibitory effects observed were due solely to AC, because crude urea extracts or culture supernatants were used which may contain PT. Recent reports by several investigators have shown that PT has various inhibitory effects on PMN and macrophage functions (1, 14, 21, 23). These include inhibition of arachidonic acid and enzyme release (2, 23), Na^+ influx and pH increase (31), superoxide generation (23), and inhibition of phagocyte CT (1, 14, 21). The present study shows that PT had significant inhibitory effects on CT and little effect on the CL response of human PMN (Table 2). Some of the effects previously reported as due to AC in crude preparations may be due to PT or to an additive effect of both.

In this paper, we report the effects on neutrophil functions of an affinity-purified AC isolated by calmodulin affinity chromatography as previously described (12). The affinity-purified AC was found to be free of PT by its inability to induce leukocytosis in mice, by the absence of cell clustering in the CHO cell assay, and by negative results with Western blot analysis with affinity-purified anti-PT antibody (12).

Affinity-purified AC, like crude AC, induced increases in PMN intracellular cAMP levels (Table 1). Whereas crude AC caused a 26.7- to 67.6-fold increase in intracellular cAMP, affinity-purified AC caused a 7.6- to 23.5-fold rise at

the doses tested. Others have shown that a five-fold increase in cAMP basal levels of PMN to 10 pmol of cAMP per 10^7 cells is sufficient to impair various phagocyte functions (3, 5, 25, 32). The results presented in Table 1 demonstrate that affinity-purified AC, like the crude preparation, caused an increase in human PMN cAMP levels and may play a role in the inhibition of PMN metabolic functions as described in this report.

The CL response of human PMN was impaired by affinity-purified AC (Fig. 1A). This suggests that myeloperoxidase-dependent production of H_2O_2 was being inhibited, because luminol-enhanced CL mainly monitors this activity (7, 10). These data and the inhibition of superoxide generation (Fig. 5) suggests that AC impairs the respiratory burst response of human neutrophils.

Although the CL response was inhibited, phagocytosis of *S. aureus* still occurred (Fig. 3). Confer and Eaton (8) reported that crude urea extracts inhibited particle ingestion. This difference may be due to the inhibition of phagocytosis by other exoproducts in the crude preparation. O'Brien et al. (22) reported that anthrax edema factor, a bacterial AC (19), and the protective antigen, which is required for cell uptake, together inhibit PMN CL response by blocking phagocytosis. Thus, two bacterial ACs which are both activated by calmodulin and cause increases in PMN intracellular cAMP levels may impair phagocyte functions by different mechanisms. Cholera toxin also causes increases in PMN cAMP levels but does not impair phagocytosis (4).

Affinity-purified AC impaired both the CL and CT reponses of human PMN in a similar dose-dependent manner (Fig. 1A and B). The 50% inhibitory dose for both was 36.5 μg/ml. Although superoxide generation by PMN stimulated with *S. aureus* was also inhibited in a dose-dependent manner, the 50% inhibitory dose required was twofold higher at 71.0 μg/ml (Fig. 5). These results suggest that CL and CT are more sensitive to the effects of AC than is superoxide generation by human PMN.

Although the results show that affinity-purified AC impaired phagocyte functions, microgram levels were required to cause 50% inhibition. PT inhibited CT, with a 50% inhibitory dose of 50 ng/ml (Table 2) compared with 36.5 μg/ml for AC (Fig. 1B). This may be due to inactivation or denaturation during purification of the portion of the AC molecule required for biological activity. The other possibility is that AC may simply require higher levels to cause its biological effects. Recent studies in our laboratory suggest that part of this problem may be due to solubility. When AC, after elution from the calmodulin-Sepharose 4B column by EGTA (12), was dialyzed in buffer without Ca^{2+}, the biological activity of the preparation increased ninefold. The 50% inhibitory dose for CL was decreased to 4 μg/ml compared with 36.5 μg/ml for the preparation dialyzed in the presence of Ca^{2+}.

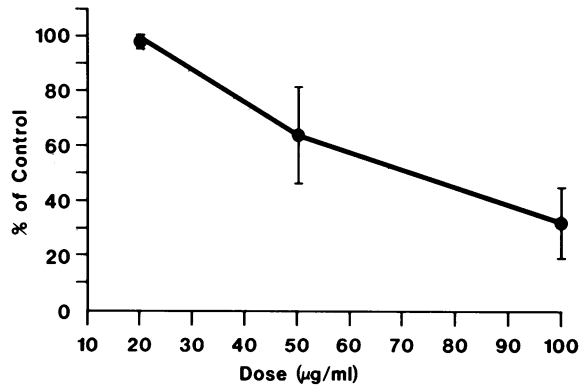

FIG. 5. Inhibition of human PMN superoxide generation by *B. pertussis* affinity-purified AC. PMN were preincubated with AC and used in superoxide generation assays using opsonized *S. aureus* as the particulate stimulant. Results are presented as the percent of control-level superoxide generation. The data are the means ± SD values from three duplicate experiments. Superoxide generation by control cells was 8.9 ± 4.3 nmol of superoxide generated per 2×10^6 PMN per 30 min.

TABLE 2. Effect of PT on CL and CT activity of human PMN[a]

PT Concn (ng/ml)	% Control ± SD	
	CL	CT
1	97.7 ± 4.6	99.2 ± 6.1
2	98.3 ± 6.5	98.0 ± 10.3
5	101.4 ± 10.1	82.3 ± 9.4
10	94.3 ± 11.0	63.5 ± 10.8
100	85.6 ± 8.9	33.3 ± 13.6

[a] PMN were preincubated with PT for 1 h at 37°C, washed, and used in CL and CT assays as described in the text. Results are the means of three duplicate experiments. CL response is given as the maximum CL intensity observed compared with control.

In summary, the results demonstrate that affinity-purified AC, which is free of contaminating PT, has biological activity. AC inhibited various human PMN metabolic functions including CL, CT, and superoxide generation, but had no effect on phagocytosis. These inhibitory effects may be due to the ability of AC to induce increases in intracellular cAMP concentration in PMN to levels which have been reported to inhibit various phagocyte functions (3, 5, 25, 32).

These results suggest that AC may be an important virulence factor in *B. pertussis* pathogenesis. AC could inhibit local host phagocytic defenses of the lung, allowing the bacteria to survive, multiply, and cause disease. AC may also be involved in the susceptibility of pertussis patients to secondary bacterial infections (30), which is a major cause of mortality associated with pertussis infections (24). Further studies are needed to confirm or deny this hypothesis.

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