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POLYMORPHONUCLEAR LEUKOCYTE-INHIBITORY FACTOR OF *BORDETELLA PERTUSSIS*. I. EXTRACTION AND PARTIAL PURIFICATION OF PHAGOCYTOSIS- AND CHEMOTAXIS-INHIBITORY ACTIVITIES

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SUMMARY A new factor that inhibited phagocytosis to opsonized targets and chemotaxis of PMN was extracted from *B. pertussis* cells, and named PMN-inhibitory factor (PIF). Cells in phase I produced 10 times more PIF than those in phase III, and like other phase I-associated components—the hemagglutinin, the histamine-sensitizing factor and agglutinogens—PIF showed degenerative, phenotypic variation during in vitro culture of phase I bacteria.

PIF was partially purified by four steps, including adsorption chromatography on Dansyl-aminononamethylene Sepharose. The resulting fraction was heterogeneous but showed little histamine-sensitizing and cytotoxic activities and was free from LPS, the hemagglutinin and a leukocyte agglutinin.

The inherent resistance of *B. pertussis* cells, in either phase I or III, as demonstrated also in the present study, and PIF-mediated defiance against immunological defense mechanism may constitute a complex host-parasite relation in experimental infections with *B. pertussis*.

INTRODUCTION

A variety of components of biological interest have been found in *Bordetella pertussis* and extensive studies have been made on the molecular basis of the pathogenesis of this infection and accompanying complications. The heat labile toxin (HLT), the hemagglutinin (HA), the histamine-sensitizing factor (HSF) and the lymphocytosis-promoting factor (LPF), with their unique biologic effects, seem to explain the mode of infection and the pathologic picture of this disease, as reviewed by Morse (1976) and Munoz and Bergman (1977). Efforts have thus been made to define a single potent protective antigen among these or other

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components, but no conclusive results have been obtained so far. Possibly a combination of two or more components forms the complete protective antigen, or some other as yet undiscovered component may also be involved.

This report describes a new factor which, like known virulent factors, is released into the culture fluid of *B. pertussis* and interferes with the phagocytic and chemotactic functions of polymorphonuclear leukocytes (PMN) of humans and animals. It seems possible that this factor, named the PMN-inhibitory factor (PIF) of *B. pertussis*, might also be related to the virulence of the organisms. The active principle was, therefore, extracted from the bacterial cells and PIF production by virulent and avirulent strains and variants of *B. pertussis* were compared. The results, in conjunction with the striking and characteristic resistance of *B. pertussis* to surface phagocytosis, suggest a complex mechanism of host-parasite relationship in this infection.

MATERIALS AND METHODS

1. *Culture medium*

Bordet-Gengou's blood agar (B-G medium) and the liquid medium of Cohen and Wheeler (C-W medium) were used in the earier part of this study. The liquid medium used in the latter part of the work was a modification of C-W medium made with reference to the reports of Sutherland and Wilkinson (1961), Morse and Bray (1969), and Parker (1976). It contained (in g per 10 liters) Casamino acid (Difco; technical), 100; cysteine, 0.4; reduced glutathione, 0.5; NaCl, 25; KH_2PO_4, 5; $MgCl_2 \cdot 6H_2O$, 1; $CaCl_2$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.005; tris(hydroxymethyl)aminomethane (Tris), 24; ascorbic acid, 0.2; nicotinamide, 0.04; soluble starch (Merck), 15; and HCl to adjust the pH to 7.2. Cysteine, glutathione, ascorbic acid and nicotinamide in a concentrated aqueous solution were filter-sterilized, and added to the autoclaved bulk of the medium.

The solid starch medium used had the same composition as the modified C-W medium, except that hydrolyzed starch (for electrophoresis; Wako Pure Chemical Industries) was added to a final concentration of 10%, in place of soluble starch. The starch was solubilized by heating with vigorous shaking before autoclaving the mixture. No agar was added.

2. *Bacteria*

B. pertussis strain Tohama (a vaccine strain; National Institute of Health, Japan) and Kendrick's 18323 were used as phase I cells, and strain Sakairi (the Kitasato Institute) was used as phase III cells. These strains were maintained on the solid starch medium. The Tohama strain at every 5–6 passage in vitro was either regenerated by passage in mouse brain, or aged by in vitro passages to provide cultures of older in vitro generations.

Bacteria grown for 24 hr on the solid starch medium were inoculated into 200 ml of the modified C-W medium and shaken for about 24 hr at 36 C. When the cell density reached $20–30 \times 10^9$ cells/ml, 20-ml portions of the subculture were transferred to 800 ml volumes of fresh medium and shaken for another 24 hr. Then phenol was added to a final concentration of 0.2% and the mixture was stored overnight at 4 C.

3. *Preparation of bacterial extract*

The bacteria were collected by centrifugation in a continuous flow system, washed with saline, suspended in 4 M urea-0.14 M NaCl-0.02 M tris(hydroxymethyl) amino methane (Tris)-HCl buffer pH 7.4 (TBS) at 0.6×10^{12} bacteria/ml and sonicated for four-consecutive 30 sec periods at 20 KHz in an Ultrasonic Disruptor (Tomy Seiko Co., Ltd.). This extract was designated as SUX.

4. *Antisera*

Agglutinins to phase I and phase III *B. pertussis* were prepared by repeated intravenous injections into rabbits of phenol-killed, washed cells of strains Tohama and Sakairi, respectively. Anti-Tohama (serotype for K-antigens; *1, 2, 4*) was made K-specific by absorption with strain Sakairi organisms. IgG antibodies were obtained by fractionation of the sera with 40% saturation of ammonium sulfate and passage of the precipitate through a DEAE-cellulose column in 0.02 M phosphate, pH 7.2.

Precipitin antibodies to the culture supernatant of strain Tohama were raised in rabbits using the zinc-precipitated fraction described by Niwa (1962) as antigen. When required, the antiserum was freed from antibody to lipopolysaccharide (LPS), as follows. LPS of phase I organisms, prepared as

described below, was adsorbed on a DEAE-cellulose column in 0.2 M phosphate buffer, pH 8. The column was washed first with the same buffer until sugar was no longer detectable in the effluent, and then with 0.04 M phosphate buffer, pH 8. A calibrated amount of antiserum was then passed through the column and the unadsorbed fraction, which was free of antibody to LPS, was collected.

Antibody to the dinitrophenyl group (DNP) was obtained by immunizing rabbits with DNP-ovalbumin in complete Freund's adjuvant. It was purified on DNP-AE-Sepharose (dinitrophenylated, ethylenediamine-coupled Sepharose 4B; Pharmacia Fine Chemicals). The antibody adsorbed on the immunoadsorbent and eluted with 2.4% dinitrophenol-NaOH, pH 8, was exclusively IgG, as judged by immunoelectrophoresis.

5. *Preparation of LPS*

LPS was extracted from *B. pertussis* cells by the hot phenol-water method of Westpbal as described by MacLennan (1960). The LPS-rich precipitate obtained by ultracentrifugation was treated with DNase, heated at 100 C for 10 min and then treated with pronase (100 μg/ml; Sigma Chemicals Co.). The final product was dialyzed first against 0.01 M EDTA and then against water, and lyophilized. The purified LPS preparation did not migrate on electrophoresis in 5% polyacrylamide gel in 1% SDS, but formed a single thick band at the top of the gel, detected by PAS-staining (Fairbanks et al., 1971).

6. *Preparation of PMN*

Human PMN were isolated from healthy donors by a modification of the method of Van Furth and Van Zwet (1973). The buffy coat layer, which contained most of the mononuclear cells was removed, and contaminating erythrocytes were lysed by incubating the preparation in 0.83% NH_4Cl, pH 7.2, for 2 min at 37 C. In the final preparation 90–95% of the cells were PMN and over 95% were viable, as judged by the Trypan blue dye exclusion test. The cells could be stored in Tris-A medium (NaCl, 7.015 g; KCl, 0.373 g; bovine serum albumin, 1 g in 1 liter of 0.02 M Tris·HCl, pH 7.4) containing 5 mM glucose for 2–4 hr without significant loss of their phagocytic activity.

7. *Surface phagocytosis of bacteria*

The capacities of PMN to phagocytize *B. pertussis* and other bacteria in the absence of opsonizing antibodies were tested on membranes to which bacteria had been fixed, by measuring the Nitroblue tetrazolium (NBT) reducing reaction of PMN (Baehner and Nathan, 1967). For the test, 5×10^8 washed bacteria were adsorbed on the surface of each filter disc (0.45 μ-pore, 13 mm-diameter; Millipore) by passing a suspension of known cell density through the filter. Then 10^6 PMN, which had been incubated with 0.02% NBT, washed and resuspended in 0.1 ml of cold Tris-A containing 2 mM KCN, were layered on each disc, and the discs were incubated in a moist chamber at 37 C for 20 min. All experiments were done in triplicate and controls with bacteria alone and PMN alone were run at the same time. After the incubation, cells were fixed with 3% glutaraldehyde and air-dried. The discs were made transparent by soaking them in xylene, and the extent of phagocytosis was estimated by scanning densitometry of the formazan derivative of NBT using a 565 nm filter. The values obtained were corrected by subtracting the values of control PMN and the respective bacterial controls. The relative phagocytosis index of PMN from an individual donor against a given bacteria was calculated from the ratio of the corrected value obtained with the bacteria to that of the same PMN with *Staphylococcus aureus*.

8. *Assay for phagocytosis inhibition*

Trinitrophenylated sheep erythrocytes (TNP-SRBC) opsonized with IgG anti-DNP were used as the standard phagocytosis inducer in the assay. One volume of a suspension of 10^9 SRBC/ml was quickly mixed with 4 volumes of 2 mg/ml 2,4,6-trinitrobenzene sulfonate (Na salt) in borate buffered saline, pH 8.5, and gently stirred for 15 min at 20 C. The cells were then washed and fixed with 4% formaldehyde. For opsonization, a suspension of 2×10^9 fixed TNP-SRBC per ml of Tris-A was mixed with an equal volume of rabbit anti-DNP (1 mg/ml) for 15 min. The multiplicity of antibody to TNP-SRBC was in large excess of that required for optimal opsonization and the cells were rapidly phagocytosed by PMN (average, 3–5 cells ingested/PMN). The phagocytic reaction of PMN and its inhibition by PIF were measured in the following two systems.

1) Measurement of O_2 consumption

In the standard assay system, samples of 10^7 PMN in 0.5 ml of Tris-A were mixed with various

dilutions of the PIF sample in a reaction vessel equipped with an oxygen-electrode, a stirrer and a water-circulation jacket (Kyusui Chemistry Lab.). The mixture was incubated for 10 min at 37 C to allow temperature equilibration and the rate of endogenous O_2 consumption was measured. Then 1×10^8 opsonized TNP-SRBC in 100 μliter, and 30 μliter of 0.1 M $CaCl_2$–$MgCl_2$ mixture were introduced to initiate the phagocytosis reaction, and the change in O_2 concentration was recorded. The rate of induced O_2 consumption, $-dO_2/dt$ in nmoles/min/10^7 PMN, was estimated from the difference between the slopes of endogenous and phagocytosis-induced O_2 consumptions. The phagocytosis inhibition (PI) activity was given by the formula,

$$\left(1-\frac{\text{Rate of } O_2 \text{ uptake in the presence of inhibitor}}{\text{Rate of } O_2 \text{ uptake in the absence of inhibitor}}\right)\times\text{sample dilution}$$

2) Measurement of the NBT reduction reaction

PMN (10^7/ml) were treated with 0.05% NBT at 37 C for 10 min and then with KCN at a final concentration of 2 mM. Serial dilutions of the test sample (25 μliter) were mixed with the treated PMN (25 μliter) in a transparent microtray with U-type wells. The mixtures were incubated for 10 min at 37 C, and then 25 μliter of opsonized TNP-SRBC, 2×10^8/ml Tris-A containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, were added. After the reaction period of 20 min at 37 C the blue color of the cells was observed, and the maximum dilution of the sample that inhibited the reaction nearly completely was taken as the PI value in units/ml.

9. *Assay of inhibition of chemotaxis*

Inhibition of chemotaxis (CI) was measured on agarose-gel plates as described by Nelson et al. (1975). A fresh culture filtrate of either *E. coli* (strain O55; K59) or *Salmonella enteritidis* was used as the chemotaxis inducing agent in most experiments. Agarose-gel at 0.7% (w/v) was prepared in Hanks' culture medium containing 10% (v/v) human serum that had been inactivated at 56 C for 30 min. PMN, 10^5 in 10 μliter, were placed in the center wells of three-well arrays and allowed to settle, and then the supernatant fluid was replaced by 10 μliter of serial dilutions of the test sample or Hanks' medium as a control. The two peripheral wells received either the chemotactic factor or medium only. Plates were incubated at 37 C in a humidified atmosphere of 5% CO_2 in air for 2–4 hr. They were then covered with 2% glutaraldehyde, and observed under an inverted phase-contrast microscope. The width of the crescent formed by the swarm of PMN migrating toward the well containing the chemotactic factor was measured by eye and the titer of the sample giving width of about half, or less than half, of that of control PMN was scored as 1 CI unit.

10. *Assay for LPF and HSF*

Lymphocytosis-promoting (LP) activity (Morse and Bray, 1969) was assayed as described by Sato and Arai (1972), and histamine-sensitizing (HS) activity was measured by the method of Lehrer et al. (1974).

11. *Hemagglutinin and passive hemagglutination tests*

Hemagglutinin (HA) activity (Keogh and North, 1948; Masry, 1952) was assayed in microtrays with U-type wells by measuring the reaction between serial two-fold dilutions of sample and a 1% suspension of SRBC. The relative amounts of cytophilic antigens, including HA and probably other antigenic components capable of binding to erythrocytes, were assayed by a passive hemagglutination method (PHA). Serial dilutions of the sample in the microtray were mixed with 1% SRBC in 2 mM EDTA-phosphate buffered saline and after 30 min at 37 C rabbit antiserum to Tohama culture supernatant that had been absorbed with LPS was added.

12. *Measurement of LPS*

LPS was measured by the hemagglutination-inhibition test using LPS-coated SRBC as described by Wardlaw and Jakus (1966). SRBC were coated with LPS at a ratio of 20 μg LPS/10^8 SRBC, and anti-Sakairi that had been absorbed with SRBC was used as the anti-LPS reagent.

13. *Analytical methods*

Approximate bacterial concentrations were estimated by the optical densities of suspensions at 650 nm using international opacity unit-coefficients of 1×10^9/ml for *B. pertussis*; 0.1×10^9/ml for *Salmonella*; 0.08×10^9/ml for *E. coli* and *Staphylococcus*; and 0.065×10^9/ml for *Streptococcus*. Protein was measured with Folin phenol-reagent as described by Lowry et al. (1951). For determination of carbohydrate, samples were hydrolyzed with 2 N HCl at 100 C for 2–4 hr in vacuum-sealed test tubes and

then HCl was removed by evaporation. Total sugar was measured by the phenol-sulfuric acid method (Dubois et al., 1956), and hexosamine by the method of Rondle and Morgan (1955). Before determination of neutral sugar the HCl-free hydrolysate was passed through a Dowex 50×8 (H^+) column in distilled water. Free fatty acids and phospholipids were determined by the methods of Itaya and Ui (1965) and Bartlett (1959), respectively.

RESULTS

1. *Surface phagocytosis of B. pertussis by human PMN*

The capacity of human PMN to phagocytose *B. pertussis* cells in the absence of opsonizing antibody was first examined with the surface phagocytosis system described in the *Materials and Methods*. In this system PMN should come in direct contact with bacteria immobilized on the rough surface of the filter membrane. Even under such favorable conditions, the susceptibility of *B. pertussis* to PMN was surprisingly low, and PMN from different healthy individuals invariably showed little phagocytosis of *B. pertussis* regardless of the phase of the bacteria (Table 1). Increase in the bacterial density from the routine number of 5×10^8 to 4×10^9 per disc did not increase the phagocytosis of strain Tohama by PMN (Table 1).

This low phagocytosis of *B. pertussis* by PMN was reflected in the fact that the bacteria did not cause increased O_2 uptake by PMN (Fig. 1). The reaction conditions in the O_2-electrode system, described in the *Materials and Methods*, may not be favorable for surface phagocytosis, but, unlike *B. pertussis*, both *S. aureus* (Fig. 1*c*) and *E. coli* (data not shown) caused marked O_2 uptake by PMN.

B. pertussis cells were, however, readily opsonized and, as shown in Fig. 1 (*a*, *b*), phagocytosis by PMN started soon after addition of the IgG fraction of rabbit antibody specific to the strain. The opsonin titers of these antibodies were nearly equal to their agglutinin titers in the homologous system, but both opsonization and agglutination showed strict strain specificity. Thus anti-Tohama could not opsonize strain Sakairi organisms and *vice versa*.

2. *Inhibitory effect of B. pertussis extract on phagocytic reactions of PMN*

Besides showing low susceptibility to phago-

TABLE 1. *Surface phagocytosis of B. pertussis and other bacteria by human PMN*

Bacteria	PMN-donor				
	T.I.	M.K.	S.U.	J.M.	S.S.
S. aureus	1.0±0.2[a]	1.0±0.3	1.0±0.0	1.0±0.1	1.0±0.2
Sal. enteritidis	1.0±0.1	0.6±0.1	ND[c]	0.6±0.1	0.5±0.1
E. coli	1.1±0.3	0.5±0.0	0.7±0.0	0.7	0.3±0.0
β-hemolytic *Strepto.*	ND[c]	0.7±0.0	ND	ND	ND
B. pertussis (phase)					
Strain Sakairi (III)	0.2±0.1	0.0±0.2	0.3±0.1	0.0±0.1	0.1±0.1
Strain Tohama (I)	0.1±0.1	0.0±0.0	0.2±0.2	0.2±0.1	0.0±0.2
Strain Tohama, ×8[b]	0.0±0.0	0.1±0.0	ND	ND	ND

[a] The relative phagocytic activity of PMN from each donor on each bacteria is expressed as the ratio of the NBT reaction with the organism to the NBT reaction of the same PMN with *S. aureus*. For details of experimental procedures, see *Materials and Methods*.

[b] The number of the bacteria was increased from 5×10^8 in the standard procedure to 4×10^9.

[c] Not determined.

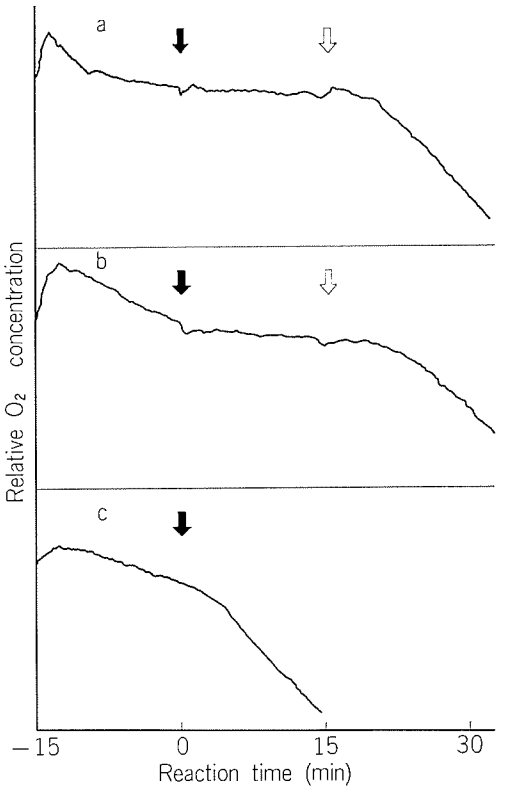


FIGURE 1 O_2 consumption of human PMN with *B. pertussis* strain Sakairi (a), strain Tohama (b), and *S. aureus* (c), in the absence or presence of opsonizing antibodies. The O_2 electrode vessel contained 1×10^7 PMN in Tris-A - 1 mM KCN. Samples of 5×10^9 cells of either strain of *B. pertussis* or 5×10^8 cells of *S. aureus* were introduced at the time indicated by ⬇, and the IgG fraction of rabbit anti-Sakairi (a) or anti-Tohama (b) at ⇩.

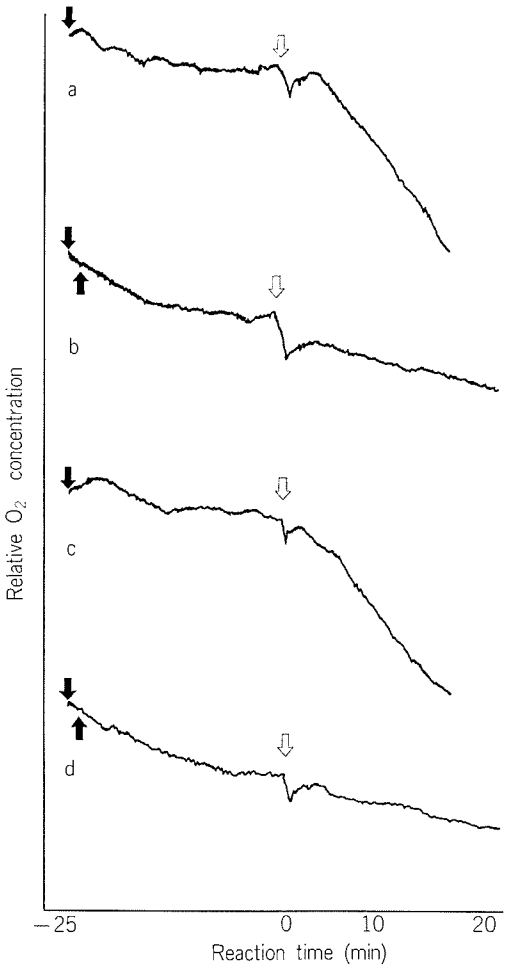


FIGURE 2 Inhibition of phagocytosis of opsonized *B. pertussis* by PIF. (a) O_2 uptake by PMN (10^7 cells) in their phagocytic response to *B. pertussis* strain Tohama opsonized with 6 units of IgG anti-Tohama. (b) The same reaction as (a) but in the presence of 0.4 ml SUX. (c) The reaction of PMN with bacteria opsonized with 12 units of IgG anti-Tohama. (d) The same reaction as (c) but in the presence of 0.4 ml SUX. PMN and SUX were added to the reaction vessel at ⬇ and at ⬆, respectively, and the opsonized bacteria at ⇩.

cytosis, *B. pertussis* releases a factor that inhibits the phagocytic activity of PMN. This effect was first observed with a culture filtrate that had been concentrated by precipitation with zinc acetate by the procedure used for preparation of HSF by Niwa (1962). Later a sonic extract in urea (SUX) was used as a source of this factor, as described in the *Materials and Methods*. For the results in Fig. 2, SUX obtained from a fresh culture of strain Tohama was dialyzed against TBS and the large amount of precipitate formed was removed by centrifugation.

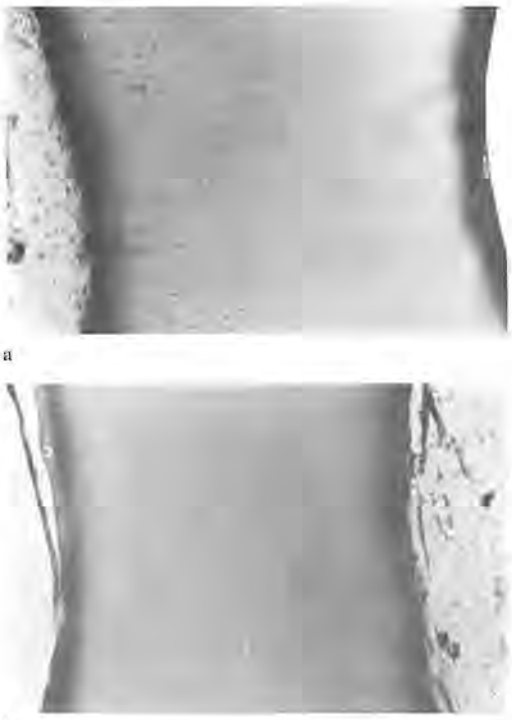
Treatment of human PMN with this clear supernatant of SUX caused marked suppres-

sion of their subsequent phagocytosis of opsonized *B. pertussis* cells. When cells of strain Tohama were treated with 6 opsonizing units of IgG anti-Tohama, 6-times the minimum opsonizing dose, 5×10^9 cells caused a net velocity of O_2 uptake of PMN, $-\frac{dO_2}{dt}$, of 32 nmoles/min/10^7 PMN (Fig. 2*a*). This value was reduced to 6.6 nmoles/min/10^7 PMN (21% of the normal response) by treatment of the PMN with 0.4 ml of SUX (Fig. 2*b*). The phagocytic response to cells opsonized with 12 units of antibody was also suppressed to the same extent (21%) by SUX (Fig. 2*c* and *d*). Therefore, the suppression of phagocytosis is independent of the amount of opsonizing antibody and thus is not due to competitive inhibition of opsonization by antigens in SUX derived from the same bacteria.

Extensive washing of SUX-treated PMN did not restore their phagocytic activity, indicating that the inhibitory factor in SUX acts directly on PMN, rather than acting on the bacteria in some way that protects them from phagocytosis. SUX also inhibited the phagocytosis of a variety of unrelated targets, including polystyrene latex particles, regardless of whether these targets had been opsonized. Therefore, studies were made on the inhibitory effect on phagocytosis of TNP-SRBC that had been opsonized with IgG anti-DNP.

3. *Effect of B. pertussis extract on chemotaxis of PMN*

SUX of strain Tohama was also found to inhibit chemotaxis of PMN toward chemotactic factors of complement, casein hydrolysate, and various bacterial origins. For instance, as shown in Fig. 3, the normal migration of human PMN toward the culture filtrate of *E. coli* was completely inhibited by the presence of SUX from strain Tohama. No clumping of cells was observed that could explain this phenomenon. Though SUX in its crude form contains weak leukocyte-agglutinating activity as well as HA, the chemotaxis-inhibition (CI) activity could still be detected at dilutions of



a

b

FIGURE 3 Chemotaxis-inhibition by *B. pertussis* extract. Normal migration of human PMN (left well) to *E. coli* chemotactic factor (right well) in (a) is completely suppressed by addition of *B. pertussis* extract (SUX) to the PMN in (b).

SUX where these agglutinins were no longer effective. Furthermore, partial purification of SUX as described below removed the agglutinin activities from the CI activity.

Although no conclusive evidence was obtained as to whether or not the CI activity was attributed to the same molecular entity as the phagocytosis-inhibition (PI) activity, "PIF" —PMN-inhibitory factor—was used as a common term for these two activities.

4. *Non-cytotoxic nature of PIF*

As is shown in Fig. 2, addition of SUX did not affect the endogenous respiration of PMN cells. The following experiment shows that CI activity was not due to a cytotoxic principle in SUX such as HLT of *B. pertussis*. PMN

were suspended in serial dilutions of SUX in Hanks' medium containing 10% inactivated human serum, and duplicate samples were incubated at 37 C in test tubes and in the CI assay system with *E. coli* chemotactic factor. After 2 hr the samples in tubes were examined by the Trypan blue dye exclusion method. At all concentrations of SUX tested, containing 0–16 CI units, less than 5% of the total cells died. It was also found (Table 2) that PIF was resistant to trypsin, whereas HLT is known to be susceptible to the enzyme (Banerjea and Munoz, 1962). The PI activity of SUX was also not due to cytotoxicity, because 16 units of SUX or less was sufficient to inhibit the phagocytic activity of PMN in the PI assay systems described above.

5. *Effects of various treatments on PIF*

Table 2 shows the effects of various chemical and physical treatments of crude and partially purified preparations of SUX on PIF. In general, the CI and PI activities in the crude SUX changed in parallel, although their behaviors showed slight differences, which can probably be attributed to inaccuracy of the assay methods. Both activities were fairly heat labile, and no activity was recovered by extraction with urea or freezing and thawing from the precipitates formed by heat treatment.

These activities were apparently resistant to trypsin and pronase, and to lysozyme in the presence of EDTA. However, treatment with pronase at 100 μg/ml (inclusive of the expander starch; Sigma) for 2–4 hr at 37 C caused a definite decrease in size of the factor with PI activity, as demonstrated by the Sephadex G-25 gel-filtration pattern in Fig. 4. Similar results were obtained with partially purified SUX. Unlike PIF, the activities of HSF and HA or PHA decreased significantly after the pronase treatment, as shown in Table 2. Pronase itself had no biologic effects.

The instability of PIF in solution and its observed action on PMN suggest that it is hydrophobic and cytophilic, and thus is adsorbed to the erythrocyte membrane by nonspecific lipophilic binding. In support of this idea, the PI and CI activities in crude SUX

TABLE 2. *Effects of various treatments on biologic activities of B. pertussis extract*[a]

Treatment	Crude extract					Partially purified
	PI	CI	HSF	HA	PHA	PI
Untreated						
Activity units	8	64	16	32	128	4
Relative activity	1	1	1	1	1	1
56 C, 30 min	1/8	1/4	1/8	1/4	1/4	1/4
100 C, 10 min	0	0	0	0	1/4	0
EDTA[a]	1	1	1/2	1/2	1/2	1
EDTA+lysozyme[b]	1	1	1	1	1	1
Trypsin[c]	1	1	1/2	1	1	1
Pronase[d]	1	1	1/8	1/16	1/16	1
RBC absorption[e]	0	0	0	0	0	1

[a] Incubated with 2 mM EDTA, then dialyzed against TBS.

[b] Incubated with lysozyme at 200 μg/ml in the presence of 2 mM EDTA for 6 hr at 37 C, $MgCl_2$ added to 0.02 M, then dialyzed against TBS.

[c] Incubated with trypsin at 50 μg/ml at 37 C for 6 hr.

[d] Incubated with pronase at 100 μg/ml at 37 C for 6 hr.

[e] Samples were re-cycled through a column of glutaraldehyde-fixed fowl or sheep erythrocytes.

were readily removed by repeated absorption with either chicken or sheep erythrocytes, as shown in Table 2. In marked contrast, however, the PI activity of partially purified preparations was not adsorbed on erythrocytes (Table 2).

6. *Partial purification*

1) Removal of nucleic acids by protamine-precipitation

A 1% solution of protamine sulfate (salmine) was added to SUX in 4 M urea-TBS until the maximum precipitation was obtained (usually at a final concentration of about 0.02% protamine sulfate) and the precipitate formed was removed by centrifugation at 15,000×*g* for 30 min. The presence of 4 M urea did not interfere with formation of the complex.

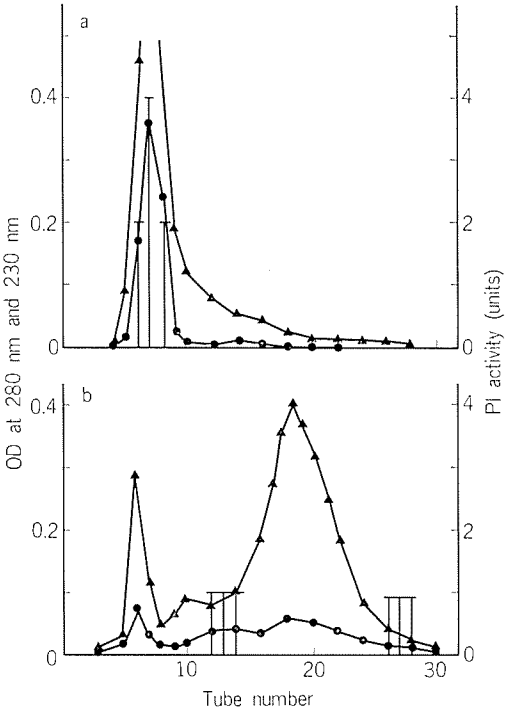


FIGURE 4 Gel filtration of the phagocytosis-inhibition activity on a Sephadex G-25 column before (a) and after (b) digestion of SUX with pronase. -●-, OD at 280 nm; -▲-, OD at 230 nm; bars, relative PI activities in arbitrary units measured by the NBT method.

2) Removal of protamine and basic proteins on CM-cellulose

The excess protamine, which caused hemagglutination at a concentration as low as 0.001%, and other basic nucleoproteins of bacterial origin were removed by CM-cellulose treatment. CM-cellulose (Na^+), which had been equilibrated with 4 M urea-TBS and drained by filtration, was added to the supernatant solution described above to give a semi-solid paste. The mixture was stirred for 20 min, and then filtered through a glass-filter. The CM-cellulose was washed with the same solvent and the washing fluid was combined with the filtrate.

CM-cellulose treatment at lower salt concentrations than that in TBS (0.14 M NaCl–0.02 M Tris·HCl) resulted in lower recovery of PIF, while omission of urea resulted in incomplete removal of protamine, presumably because of its association with some acidic components. These two steps together gave nearly full recoveries of PI and CI activities, but reduced the HA and PHA titers to half those in the original SUX.

3) Fractionation by ammonium sulfate precipitation

PIF was precipitated from SUX with 70% saturation of ammonium sulfate without loss of activity, but it was unstable in TBS without urea. The precipitate with 20–30% saturation of ammonium sulfate, which mostly floated upon centrifugation, was scarcely soluble in a physiological solvent, but it became soluble when combined with the precipitate at 70% saturation or with the precipitate obtained by further addition of the salt to the supernatant at 30% saturation. The strong interactions between components seem to modify their solubilities.

To obtain PIF in a soluble form, the CM-cellulose treated material, described above, in 4 M urea-TBS was adjusted to 22% saturation of ammonium sulfate (12.5 g of salt/100 ml) and the precipitate was removed. The super-

natant was then brought to 73% saturation by addition of further 37.5 g of salt/100 ml original volume and the resulting precipitate was dissolved in TBS at one-fourth the volume of the original SUX.

In these three steps the recovery of PIF was nearly 100% with respect to the activities of CI and PI, with 3- to 4-fold increase in the specific activity on the basis of the activity/protein ratio.

4) Adsorption chromatography on DNS-ANM-Sepharose

Since the main constituents of SUX were presumed to be membrane proteins, we tried to separate PIF by adsorption chromatography on a lipophilic matrix. The best result obtained so far was with Dansyl-aminononamethylene-Sepharose(DNS-ANM-Sepharose), prepared by coupling 1,9-diaminononane (10 mmoles) with BrCN-activated (Cuatrecasas, 1970) Sepharose 4B (60 g wet), and then reaction of the free amino groups at pH 11 with Dansyl chloride (4 mmoles), added dropwise as a solution in acetone. When kept in the dark, DNS-ANM-Sepharose could be used repeatedly after washing successively with 2% Triton X-100, H_2O, 10% (v/v) propionic acid, H_2O and TBS.

The ammonium sulfate fraction described above (20–50 ml) was applied to a 60 ml column of DNS-ANM-Sepharose in TBS-2 mM EDTA and the column was eluted with a gradient of urea in TBS. Figure 5 shows typical results. The PIF activity, measured by the NBT method described above, appeared in the unadsorbed fraction with tailing that indicated its considerable heterogeneity in affinity for the adsorbent. The extent of retardation on the column varied in different samples. This heterogeneity may depend on the amount of coexisting components that interact with the active moiety. Thus, the PIF activity in crude SUX was strongly adsorbed and was eluted only with concentrations of over 4 M urea or with 2% Triton X-100.

As shown in Fig. 5, the PIF activity was clearly separated from HA, LPS and a leukocyte agglutinating principle, which were recovered in Fr III, Fr II and III, and Fr IV, respectively. No HSF activity was present in Fr I which contained 25–50% of the applied PIF. Fr I was concentrated by precipitation with 70% saturation of ammonium sulfate.

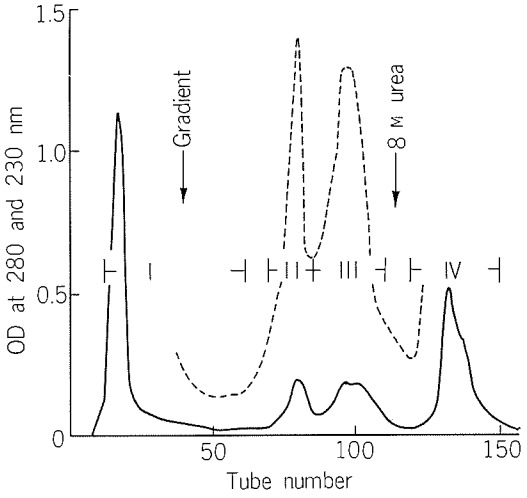


FIGURE 5 Adsorption chromatography of partially purified SUX on DNS-ANM-Sepharose. Twenty milliliter of ammonium sulfate-fractionated SUX (see Text) was applied to a 60 ml-column of DNS-ANM-Sepharose. The column was eluted successively with (1) TBS-2 mM EDTA, (2) a concentration gradient of 0 to 6 M urea in TBS, and (3) 8 M urea in TBS. The gradient was delivered from a Varigrad apparatus with 300 ml of TBS-2 mM EDTA in the first three compartments, 100 ml of 4 M urea-TBS in the fourth, and 300 ml of 6 M urea-TBS in the last three compartments. Pooled fractions I, II, III and IV were dialyzed and concentrated to 10 ml by ultrafiltration with Diaflo UM2 membranes (Amicon). Solid line, OD at 280 nm; broken line, OD at 230 nm.

7. *Relation of PIF production and degenerative changes of phase I organisms*

As originally reported by Leslie and Gardner (1931), during culture, *B. pertussis* rapidly loses the phenotypic characters of freshly isolated virulent organisms. These changes are presumably restricted to surface components, such as agglutinogens, HA, and protective

antigen, and probably also involve factors that determine virulence (Standfast, 1951). This and the difficulty we often encountered in obtaining PIF from cultures of laboratory stocks of strain Tohama prompted us to see whether PIF production also decreases during culture.

Fresh isolates from mouse brain (strain Tohama) were transferred to solid starch medium, which gave faster growth than B-G medium, and the culture medium was renewed every five days. Organisms from these stock cultures after various numbers of in vitro passages were grown in modified C-W medium, and harvested when the growth reached $20–30\times10^9$/ml. Bacterial extracts (SUX) were prepared and subjected to the protamine, CM-cellulose, and ammonium sulfate steps described above. The concentration of the products was adjusted to be equivalent to that in 2.4×10^{12} bacteria/ml. Protein in the culture supernatant was precipitated with 70% saturation of ammonium sulfate. The precipitate was dissolved in 4 M urea-TBS and subjected to treatments described above. The PI, CI,

TABLE 3. *Decrease in PIF production by B. pertussis strain Tohama by in vitro ageing*

Cultured bacteria		Agglutination[b]			Activity extract of culture filtrate				
Strain	*In vitro* passage[a]	Anti-Tohama		Anti-Sakairi		PI (Units)	CI (Units)	HA (Units)	HSF (HSD_{50})
		0.5 hr	24 hr						
Tohama	2	256	2048	<2	SUX[c]	16–8	64	32	160
					C.F.[d]		64	8	40
	4	256	2048	<2	SUX	16–8		64	80
					C.F.	4		8	40
	6	64	2048	4	SUX	8	64	32	
	10	32	512	16	SUX	4	32	8	80
					C.F.	2	16	8	40
	15	32	256	64	SUX	4	8	4	40
					C.F.	2		8	20
	20	16	32	64	SUX	2	16	8	80
					C.F.	2		8	40
	30	<16	<16		SUX	2	8	4	10
					C.F.	2	8	0	10
	40	<16	<16	256	SUX	2	8	1	20
					C.F.	2	4	0	20
Sakairi		<16	<16	512	SUX	2	4	0	<5
					C.F.	1	4	0	<5
18323	4	64	512	<2	SUX	8	32	16	80
	25	32	256	<2	SUX	8	32	16	80
					C.F.	8	32	8	80

[a] Freshly isolated bacteria from mouse brain were cultured on solid starch medium (*Materials and Methods*) and then passaged in vitro at 5 days intervals for the number of times indicated.

[b] Dilutions of antiserum were treated with 10×10^9 washed live organisms.

[c] Bacterial extract (SUX) was purified to the ammonium sulfate step (*see Text*).

[d] Material in culture supernatant precipitated at 70% ammonium sulfate saturation. Concentrations used were equivalent to an original bacterial number per ml of 2.4×10^{12}.

HA and histamine-sensitizing (HS) activities of these products were assayed and their changes with age of the cultures were examined. The results are shown in Table 3. For comparison Table 3 also shows the agglutination titers of the organisms at each age with anti-Tohama that had been absorbed by cells of strain Sakairi and with anti-Sakairi.

The results show that the PI and CI activities of PIF decreased with age of the cultures. The assay methods were not sufficiently accurate to allow us to show these changes as continuous curves, but the results clearly show a sharp fall in PIF between the 4–6th passage and the 15th passage. Similar decreases were observed in the HA and agglutination titers. After this decrease, the PIF activity appeared to remain steady at the level of phase III, strain Sakairi cells, as also shown in Table 3. These old cultures reverted to PIF producers on mouse brain passage, with similar increases in their other activities. Table 3 also shows that, in contrast to strain Tohama, strain 18323 was relatively stable in these properties.

DISCUSSION

In the surface phagocytosis assay system devised in the present study, cultures of *B. pertussis* showed very low susceptibility to surface phagocytosis by human PMN. This low susceptibility was observed with both phase I and phase III cells, and with PMN from different healthy donors (Table 1, Fig. 1). This low susceptibility cannot be ascribed to any of the main surface structures known, such as capsule or LPS, because strain Sakairi has no capsule or K agglutinogens and because the cultures of strain Tohama used in these experiments were not agglutinated by anti-Sakairi, which consists mainly of antibodies to LPS. Possibly some unidentified structure or substance, or some physical property, such as the small size, makes these organisms highly resistant to phagocytosis. On treatment with specific IgG antibodies, these cells were easily opsonized and phagocytosed by PMN cells.

From studies on mouse brain infections, Adams and Hopewell (1970) inferred that neither local phagocytes nor circulating leukocytes are capable of controlling infections with virulent strains of *B. pertussis* in unimmunized mice. In immunized mice, the bacteria could still overgrow neuroglial cells until the blood-brain barrier broke down, but they were then cleared up, presumably by the action of blood leukocytes with the aid of opsonizing antibody. Similar results in support of this concept were also reported by Holt (1972). The present findings provide direct proof for these observations.

In addition to the inherent resistance of *B. pertussis* to surface phagocytosis, we found a cell-free factor that inhibited the functions of PMN. This factor, named PIF, was detected in the culture fluid of phase I organisms of *B. pertussis*. It could also be extracted from bacterial cells by mild sonication in the presence of 4 M urea (SUX), by a procedure similar to that used for the extraction of HSF by Lehrer et al. (1974). The ready release of the PIF activity suggests that the factor may be associated with the surface structure of the bacteria; in fact, our later studies on isolated cell walls showed that PIF probably originates from the outer membrane (Imagawa et al., data to be published).

The susceptibility of PIF to heat and pronase treatments (Table 2, Fig. 4) suggests its protein nature. However, although PIF was degraded by pronase, it did not lose activity. Therefore, the possibility that PIF is a small non-protein molecule but is firmly attached to a protein carrier is not completely excluded. In contrast, HA and HSF lost their activities when treated with pronase, in agreement with the results of Lehrer et al. (1975).

The present method of purification of PIF is unsatisfactory, and the preparation consisted of several protein components, as judged by polyacrylamide-gel electrophoresis. Further purification of PIF is difficult because of its instability and physical polymorphism,

which is reflected in its inconsistent behavior on gel filtration or ion exchange cbromatography. It is interesting that HSF (Munoz et al., 1970) and HA also seem to show molecular polymorphism. Removal of poorly soluble, low density material by precipitation with low saturation (22%) of ammonium sulfate greatly changed the physical behavior of PIF. Thus, after the ammonium sulfate fractionation, PIF became more soluble in TBS without urea but at the same time lost its erythrocyte-bindability (Table 2). It is therefore possible that PIF is closely associated with some lipophilic material in the crude extract.

Extraction of lipid with an organic solvent would have reduced the molecular polymorphism but also the yield of PIF. Therefore, we used DNS-ANM-Sepharose as an adsorbent with affinity for hydrophobic substances. Cbromatography on DNS-ANM-Sepharose separated PIF from HA, LPS and a leukocyte-agglutinating activity, but gave very poor recovery for HSF. HSF may have been inactivated on the column by dissociation into subunits, which have been described for LPF (=HSF) by Morse and Morse (1976). Because of the low recovery of the HSF activity, we could not determine whether PIF and HSF are structurally related.

A close parallel was found between PIF production and other properties and products known to be characteristic of virulent cells of *B. pertussis*. This parallel was demonstrated in association with both genetic phase variation and phenotypic variation, known as modulation or mode variation of phase I organisms. Table 3 shows that PIF production by strain Tohama was subject to degenerative change and that this change was parallel with changes in the agglutination titer of the bacteria and in the HA activity of extracts. The parallel of PIF with HSF was less clear, but this may be attributed to the low accuracy of the assay. Tbe mecbanism for these continuous phenotypic changes has not been clarified. However, since HA is not identical with PIF or with HSF (Morse and Morse, 1976), it is possible that HA, HSF (LPF) and PIF are expressed on the bacterial surface as a cluster or a complex of molecules, and that the absence of one key component from the surface may influence production of the others.

In contrast to strain Tohama, strain 18323 showed a considerable stability with regard to PIF activity and other properties, as shown in Table 3. It is interesting that the virulence of tbis strain to mice is also stable in culture (Adams, 1970).

Our observations, in conjunction with those by Holt (1972), seem to be consistent with the following series of events in experimental *B. pertussis* infections. (1) The bacteria grow freely in the pre-immune host, escaping from the local surface phagocytosis. (2) IgG antibody in the immune host mediates opsonized phagocytosis by PMN, thus constituting the main host defense. (3) This defense may be inhibited by PIF-mediated defiance by the virulent bacteria. Thus it is tempting to speculate that immunologic neutralization of PIF, together with the opsonizing antibodies, reinforce the host's defense against *B. pertussis* infections.

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