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POLYMORPHONUCLEAR LEUKOCYTE-INHIBITORY FACTOR OF *BORDETELLA PERTUSSIS*. II. LOCALIZATION IN THE OUTER MEMBRANE

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SUMMARY The outer and inner membranes and cytoplasm of spheroplasts of a strain of phase I *B. pertussis* were fractionated by density gradient centrifugation. The high density vesicles of the outer membranes isolated had the "Pili" characteristic of the bacteria and the same antigenicity as the bacterial surface. Activities for inhibition of polymorphonuclear leukocytes were also almost exclusively localized in this outer membrane fraction. The histamine-sensitizing activity was more dispersed, but its specific activity was also highest in the outer membrane fraction. These results suggest that molecules carrying these activities, which are probably different entities together with the tissue-adhesive pili, form a virulence complex on the surface of phase I organisms of *B. pertussis*.

INTRODUCTION

In the preceding report we described a principle, designated as polymorphonuclear leukocyte-inhibitory factor (PIF), of *Bordetella pertussis* which interferes with the phagocytic and chemotactic functions of polymorphonuclear leukocyte (PMN) (Utsumi et al., 1978). The inhibitory effect of PIF on the host's defence mechanism, the resistance of *B. pertussis* to surface phagocytosis, the higher content of PIF in phase I than phase III organisms, and the close parallel of PIF production with production of the other components characteristic of phase I, virulent organisms, led us to speculate that PIF may have pathogenic implications in pertussis infections. Previous findings suggested that PIF may be derived from the surface of the organism, and if true, this would also support the idea of biological and immunological significance of PIF. In this work we examined the location of PIF in *B. pertussis* cells.

MATERIALS AND METHODS

1. *B. pertussis culture*

Strains Tohama and Sakairi were used as phase I

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and III organisms, respectively. Organisms were cultured in the liquid medium of Cohen and Wheeler (1946) without addition of charcoal and harvested in the late exponential phase of growth. Growth was slower in this medium (C-W medium) than in the modified C-W medium described previously (Utsumi et al., 1978), but control of growth for preparation of spheroplasts was easier.

2. *Isolation of membrane fractions*

Membrane fractions were separated essentially as described by Yamato et al. (1975). A suspension of washed cells ($2–4\times10^{11}$ cells/ml) in 20% sucrose (W/V) in 30 mM tris (hydroxymethyl) aminomethane (Tris)·HCl-10 mM EDTA, pH 8.0 was treated with lysozyme (egg white, Worthington) at a final concentration of 100 μg/ml at 0 C for 1 hr with constant stirring. The spheroplasts thus formed were disrupted in a Mühler Cell-Mill (Edmunde, Type V12) at 250 watts for 30 min in 20% sucrose-3 mM EDTA (disodium), pH 7.2. The intact cells were removed by centrifugation at $2,000\times g$ for 15 min, and the crude membranes were precipitated by centrifugation at $78,000\times g$ for 1.5 hr and washed first with 10% sucrose-3 mM EDTA and then with 3 mM EDTA without sucrose; this preparation was designated as " crude membranes ".

This crude membrane fraction was layered over 44% sucrose (W/W) in 3 mM EDTA and centrifuged at $78,000\times g$ for 12 hr at 4 C. The top layer was separated as the " M-I " fraction. The pellet was washed with 10% sucrose-3 mM EDTA, layered on a bilayer of 52% and 56% sucrose (W/W) in 3 mM EDTA and recentrifuged. The bands formed on 52% and 56% sucrose layers were separated and designated as " M-II " and " M-III ", respectively.

3. *Extraction by sonication in urea*

Whole bacterial cells or the membrane fractions were sonicated in ice-cold 4 M urea-1 M NaCl-0.1 M phosphate buffer, pH 7, at 200 watts for 2 min in an Ultrasonic Disruptor (Tomy Seiko Co., Ltd.) and the extract, designated as SUX, was dialysed against 0.14 M NaCl-0.02 M Tris·HCl, pH 7.4 (TBS).

4. *Assay for PMN-inhibitory activities*

PIF was assayed by measuring chemotaxis-inhibition (CI) and phagocytosis-inhibition (PI) as described previously (Utsumi et al., 1978). Briefly, dilutions of sample were mixed with an equal volume of freshly isolated human PMN cells at a concentration 1×10^7/ml in a micro-titration plate, and the treated cells were tested for chemotactic activity to *E. coli* culture filtrate in modified Nelson's agarose plates (Nelson et al., 1975).

For the PI test, PMN cells that had been treated with 0.2% Nitroblue tetrazolium, were treated with samples as above, and then this phagocytic activity to IgG antibody-opsonized trinitrophenylated sheep erythrocytes in the presence of 2 mM KCN was judged by the blue color of functioning cells. The reciprocals of the maximum dilutions that prevented migration and the blue color in the respective assay systems were taken as the CI units, and PI units per ml of sample.

5. *Immunofluorescence staining*

Antisera to the membrane fractions M-I and M-III were raised in rabbits by two injections of 2 mg protein of these fractions in Freund's complete adjuvant with a one month interval between injections. The IgG fraction of guinea pig anti-rabbit IgG was prepared by ammonium sulfate precipitation and then DEAE-cellulose chromatography and was conjugated with fluorescein isothiocyanate (FITC). Thin smears of phase I bacteria were treated with the respective antisera at 37 C for 10 min, washed thoroughly with saline, and stained with FITC-anti-rabbit IgG.

6 *Electron microscopy*

Specimens on hydrophilic grids were negatively stained with 1% uranyl acetate, pH 4.3, and observations were made in a JEOL 100B electron microscope. The shadowed profiles were observed using platinum-palladium alloy

7. *Analytical methods*

Protein was determined by the method of Lowry et al. (1951) and total sugar was assayed by the method of Dubois et al. (1956) with glucose as standard without previous hydrolysis of the samples. 2-Keto-3-deoxyoctonate (KDO) was determined by the method of Osborn et al. (1972).

RESULTS

1. *Physical and chemical properties of membrane fractions*

The density distribution of the spheroplast

membrane fractions from *B. pertussis* phase I (strain Tohama), which were obtained by the procedures used for *E. coli* by Yamato et al. (1975), was examined by density gradient centrifugation. As shown in Fig. 1 the three fractions formed distinct boundaries in a gradient of 35.5% to 50% sucrose. The M-I fraction gave contiguous multiple peaks, the heaviest of which contained the highest activity of Mg^{++}-dependent ATPase. The bulk of the M-III fraction was precipitated to the bottom on 4-hr centrifugation at 95,000×*g*, but some lighter component which coincided with the peak of M-II was also present. Fraction M-II was of intermediate density.

In general this pattern resembles that of spheroplast membranes of *Salmonella typhimurium* described by Osborn et al. (1972), who identified the heterogeneous boundary of lower density and the fraction of greatest density were identified as cytoplasmic membranes and the outer membranes, respectively. A similar density distribution has also been reported for the spheroplast membranes of *Escherichia coli* (Yamato et al., 1975; Mizushima and Yamada, 1975). The boundary of M-II of intermediate density may be a complex of the two membranes, or correspond to murein-outer membrane complexes. The ratio of the yields in dry weight of M-I: M-II: M-III in a typical preparation was approximately 4: 4: 2.

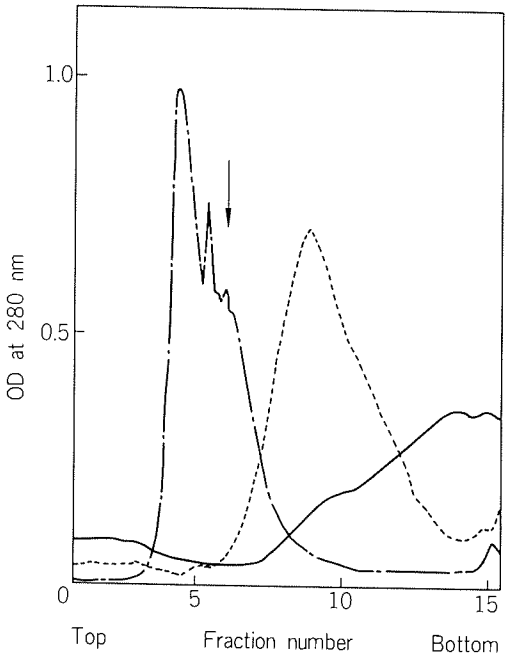
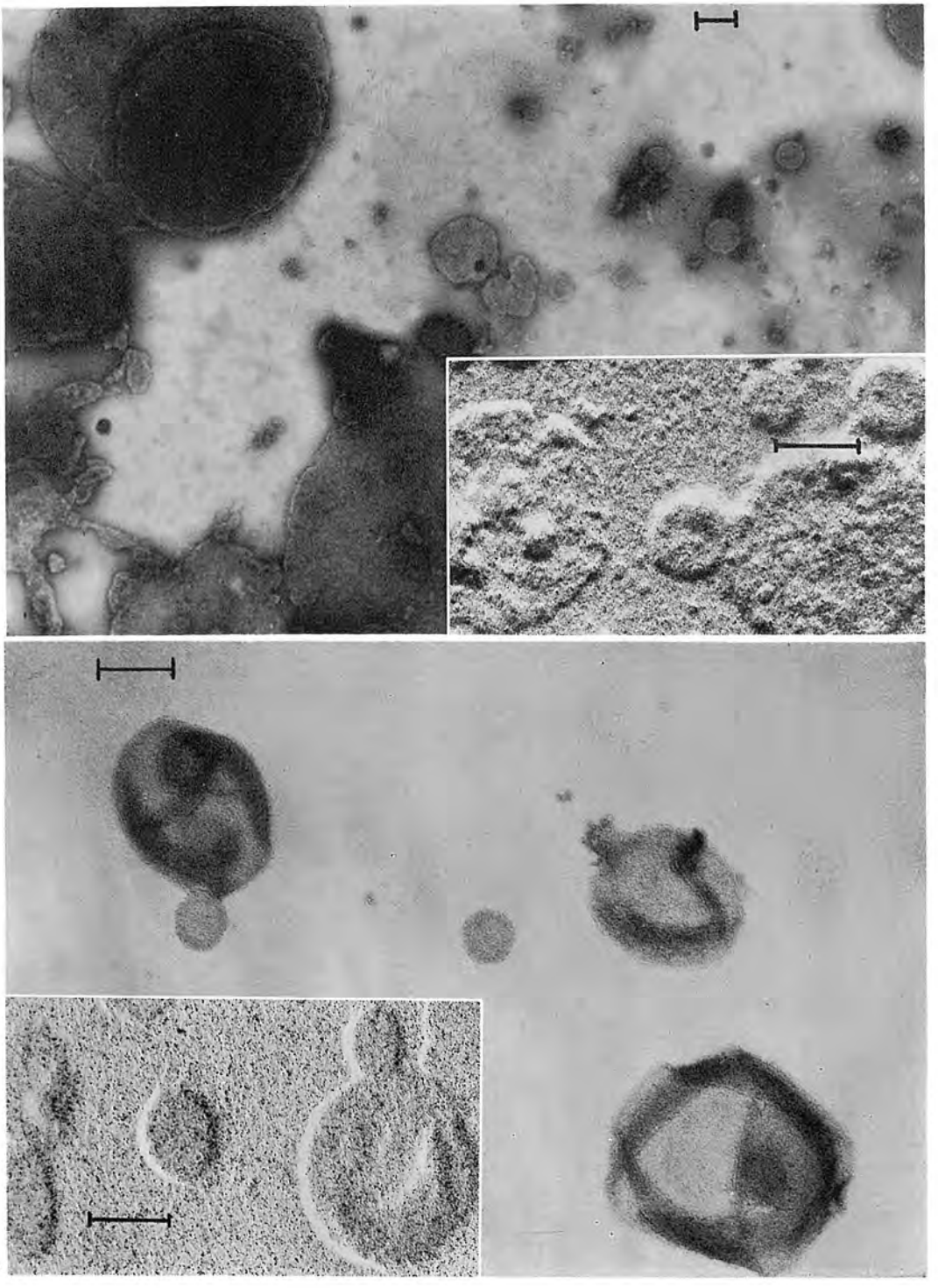


FIGURE 1. Distribution of spheroplast membrane fractions of *B. pertussis* on density gradient centrifugation. Fractions M-I (—·—), M-II (- - -), and M-III (——) prepared from phase I strain Tohama as described in the *Materials and Methods* were centrifuged separately on linear concentration gradients of 35.5% to 50% sucrose (w/w) at 4 C and 95,000×*g* for 4 hr. The arrow indicates the position of ATPase activity.

The electron microscopic appearance of M-I (Fig. 2a) was in many respects characteristic of inner (cytoplasmic) membrane vesicles; the membranes were heterogeneous in both size and shape with an irregular contour and granular surface. The M-II fraction (Fig. 2b) resembled the envelopes consisting of murein layer and outer membrane obtained by mechanical disruption of whole cells of *E. coli* (Schnaitman, 1970). In marked contrast to these fractions, fraction M-III (Fig. 2c) appeared as smooth, flatten, empty and sometimes coiled vesicles of fairly homogeneous size. The appearance of these vesicles was similar to that of outer membranes of *E. coli* (Yamato et al., 1975: Mizushima and Yamada, 1975), but unlike in preparations from *E. coli*, a number of filamentous structures were also seen attached to, and extended outward from, the vesicle surface (Fig. 2c). Similar structures have been found on the cell wall of phase I organisms of *B. pertussis* (Morse and Morse, 1970) and designated as "pili" (Morse and Morse, 1976).

The higher carbohydrate content, and lower protein and cytochrome contents of M-III (Table 1) than of M-I, are indicative of its outer membrane origin, but its lipopolysaccharide (LPS) content estimated by KDO was low, because of the peculiarly low LPS content in *B. pertussis* (approximately 0.2%).



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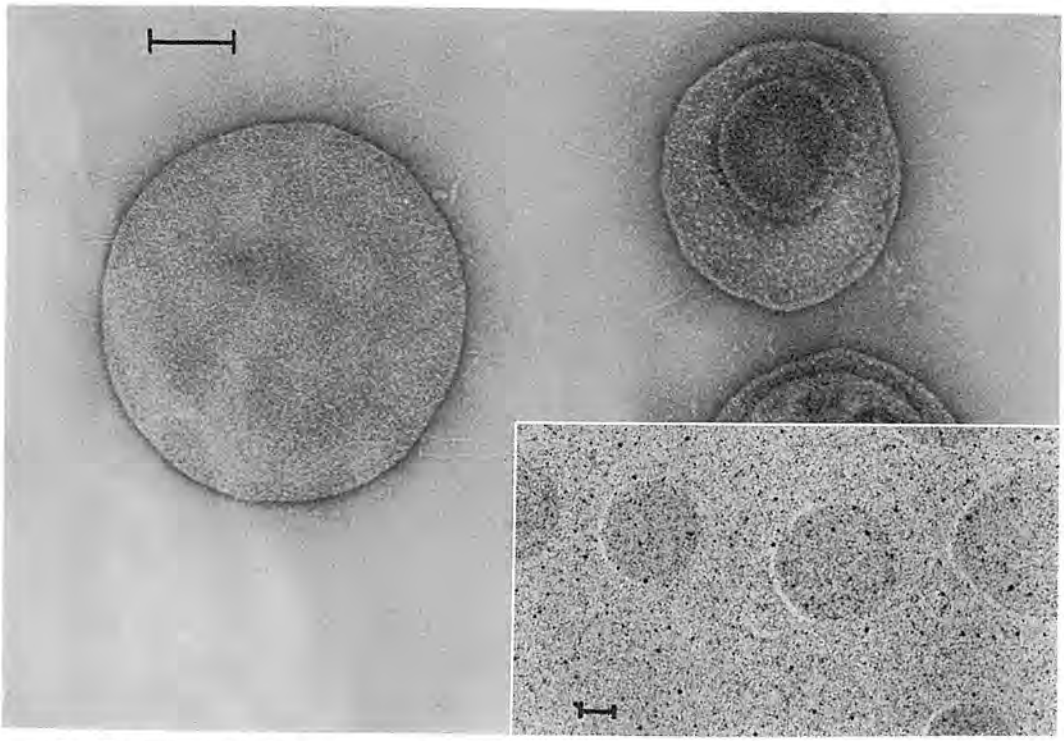

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FIGURE 2. Electron microscopic appearance of spheroplast membranes of *B. pertussis*. Membrane fractions, (a) M-I (cytoplasmic membranes), (b) M-II (membrane complexes), and (c) M-III (outer membranes), prepared from phase I strain Tohama, were negatively stained with 1% uranyl acetate. Note the filamentous structures on M-III vesicles (c). Insets illustrate shadowed profiles. Scale: 0.1 μm.

TABLE 1. *Protein, Carbohydrate and cytochrome contents*

	% Dry weight		Cytochromes[a] OD^{561} nm
	Protein	Sugar	
M-I	68.7	4.0	0.138
M-II	58.1	5.9	0.060
M-III	55.5	11.7	0.020

[a] Difference in absorbance at 561 nm between hydrosulfite-reduced and ferricyanide-oxidized samples at 10 mg protein/ml.

2. *Reactivity of anti-membrane antibodies with the bacterial surface*

When a fesh culture of strain Tohama was treated with rabbit anti-sera to M-I (Fig. 3a) and M-III then with FITC-labelled anti-rabbit IgG, as described in the *Materials and Methods* (Fig. 3a and b), only the anti-M-III treated cells appeared fluorescent (Fig. 3b). This staining was specifically blocked by treatment of the anti-M-III-coated bacteria with unconjugated anti-IgG before FITC-labelled antibody (Fig. 3c).

a

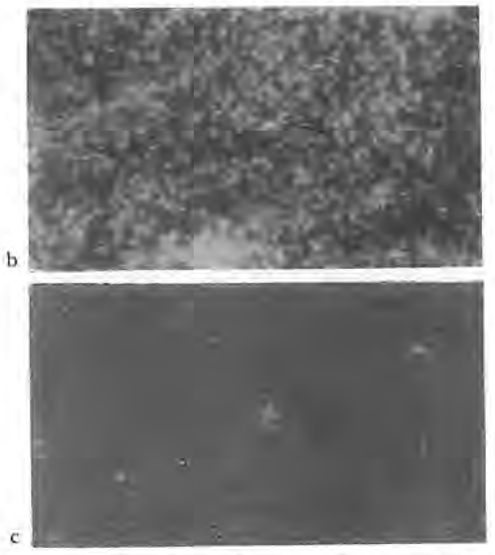

FIGURE 3. Reactivity of anti-membrane antibodies with the bacterial surface of *B. pertussis*, phase 1 strain Tohama. (a) Cells were treated with anti-M-I and then with FITC-labelled anti-IgG. (b) Cells were treated with anti-M-III and then with FITC-labelled anti-IgG. (c) Cells were coated with anti-M-III as for (b), and with unconjugated anti-IgG and then treated with FITC-labelled anti-IgG.

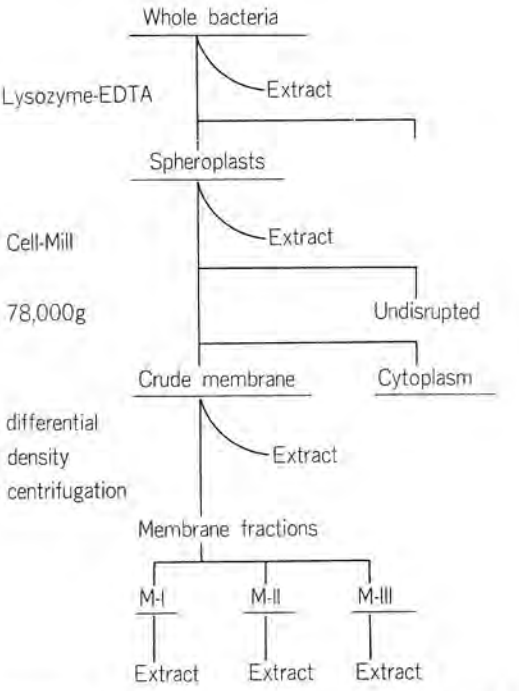

FIGURE 4. Scheme of subcellular fractionation and extraction. Details of the procedures are described in the *Materials and Methods*.

3. *Localization of PIF and HSF activities*

The locations of PIF and HSF during fractionation of subcellular components of fresh cultures of strain Tohama were next investigated as illustrated in Fig. 4. For determination of the activities in whole bacterial cells, spheroplasts, and membrane fractions, the preparations were extracted with 4 M urea-1 M NaCl at neutrality by mild ultrasonic treatment and the extracts were dialysed against Tris·HCl-saline, pH 7.4, before assay, as described in *Materials and Methods*. For comparison, the cytoplasmic sap was also treated in the same manner. The PIF contents of these extracts were determined by simultaneous assays of their activities for chemotaxis-inhibition (CI) and phagocytosis-inhibition (PI) on freshly isolated human PMN cells from a single healthy donor.

The localization of PIF activity in the cell wall compartment was evidenced by comparison of the crude membrane and cytoplasm fractions with the original whole bacterial cells; the activity found in the whole bacteria was almost fully recovered in the spheroplasts, and 60–70% of the activity was recovered in the crude membrane fraction, whereas only 6% or less was present in the cytoplasmic sap. Another 6% of the original activity was attributed to undisrupted spheroplasts. The disruption of spheroplast-associated PIF among the membrane fractions, obtained in one experiment, is shown in Table 2. The high content of PIF in the M-III fraction was indicated by its high specific activity and high recovery in this fraction, measured as CI activity. PI activity (not listed in Table 2) was essentially parallel to CI activity and the same preparations of M-I and M-III as in Table 2

TABLE 2. *Localization of PMN-inhibitory and histamine-sensitizing activities*

	PMN-inhibitory activity		Histamine-sensitizing activity	
	CI units[a] per mg protein	Recovery %	HSD_{50}[b] per mg protein	Recovery %
Spheroplasts[c]	1.08	100	117	100
Crude membranes[d]	2.11	65	370	52
Fraction M-I[e]	1.29	12.4	480	21.9
Fraction M-II	2.56	22	340	13
Fraction M-III	12.1	51.7	950	18.6

[a] One CI unit is defined as the activity causing nearly complete inhibition of chemotaxis of 10^7 PMN. Dilutions of sample were mixed with 10^7/ml PMN (1 : 1) in microtiter plates and the treated cells were tested for chemotactic activity to *E. coli* culture filtrate in agarose chemotaxis plates of Nelson et al. (1975).

[b] Groups of mice were injected i.v. with dilutions of samples that had been heated at 56 C for 10 min and challenged 3 days later with 2 mg histamine. The LD_{50} value as the HSD_{50} was estimated from the number of deaths within 16 hr (Lehrer et al., 1974).

[c] Spheroplasts from strain Tohama organisms, and the extract for assay, obtained by sonication in urea, were prepared as described in the *Materials anh Methods.* The extract was dialyzed before assay.

[d] Unfractionated membrane complexes of disrupted spheroplasts were extracted and dialyzed before assay.

[e] Membrane fractions separated by differential density centrifugation as described in the Materials and Methods were extracted and dialyzed before assay.

showed 0.7 and 12.1 PI units per mg protein, respectively. To other similar experiments gave speific CI activities of 13.8 and 15.5 units per mg protein in M-III preparations with 0.6 and 2.4 units per mg protein, respectively in the corresponding M-I preparations. The specific activity of M-II was always intermediate between those in fractions M-I and M-III. Little PIF activity was found in the M-III fraction unless it was solubilized; the CI activity of M-III in its insoluble vesicular form was only one-sixteenth of that found in the extract. In contrast, untreated M-I was as active as the extract from it. The untreated M-II fraction also contained an active form of PIF, but this amounted to half the total activity found in the extract, again indicating the intermediate property of this fraction.

Histamine-sensitizing (HS) activity was more widely distributed and could not be exactly localized like PIF (Table 2). About one-third of the activity found in the extract of spheroplasts appeared in the cytoplasmic sap after disruption, and the envelope-associated activity (crude membranes, Table 2) too was distributed between the membrane fractions. Nevertheless, the specific activity was 3-fold higher in the crude membranes, and 8-fold higher in the M-III fraction than in original spheroplasts.

DISCUSSION

The method used for separation of membrane fractions of *E. coli* by Yamato et al. (1975) proved useful in the present study on *B. pertussis.* The yield of our M-III fraction, which corresponds to their outer membrane fraction, however, was not quite satisfactory (about 20% of the entire spheroplast membranes), indicating that improvement in the experimental conditions are necessary. Decomposition of the peptidoglycan may have been incomplete resulting in a poor recovery of the outer membranes and a relatively high yield of the intermediate fraction, M-II, which may consist of entire cell walls or the murein-outer membrane complexes, or both.

The density distribution, the electron microscopic appearance particularly the characteristic "pili", the serologic activity, which was the same as that of the bacterial surface, and chemical findings all indicate that M-III fraction was derived from the outer membranes. The purity of M-III could not be estimated, but the presence of inner membranes or other atypical aggregates would have been detectable under an electron microscope, and the presence of soluble molecules in this vesicular fraction seems unlikely, unless these molecules are secondarily adsorbed. On the other hand, the main inner membrane fraction M-I may well be contaminated by outer membranes as well as soluble components. The biologic principle, PIF, was previously found to be released spontaneously into the medium and to be readily extractable from whole cells of phase I *B. pertussis* by mild procedures (Utsumi et al., 1978). As shown in Table 2, in this work, analysis of the inhibitory effects of subcellular components on PMN showed that PIF was localized in the outer membrane fraction (M-III). Most of the activity in the whole bacteria was retained in the spheroplasts and little, if any, was found in the cytoplasm after mechanical disruption of the spheroplasts. Most of the activity retained by the spheroplast membranes was recovered in the outer membrane fraction. The marked increase in the specific activity of PIF in the M-III fraction, 11 times that in the spheroplasts, (Table 2) supports the conclusion that PIF is attached exclusively to the outer membranes.

The PIF associated with the outer membrane fraction was not effective. This suggests that PIF is incorporated into a macromolecular assembly on the membrane, in a masked or immobilized state. The small proportion (6%) of activity found in the untreated outer membrane fraction might therefore indicate a slow, spontaneous release of PIF molecules into the medium. In contrast, all the activity in the inner membrane fraction was present in an active form, and is probably due to contamination of this fraction with free PIF molecules liberated from the outer membrane during the isolation procedure.

The histamine-sensitizing effect had a wide distribution, and consequently the exact location of HSF could not be determined in the present study. This wide distribution may in part be due to technical difficulties in estimating HS activity which is elicited by HSF in the presence of other biologically active components, such as lethal toxins of *B. pertussis*. The results in Table 2 were thus obtained by assays on samples that had been heated at 56 C for 10 min to inactivate heat-labile toxin, but this heat treatment may have lowered the HS activity to different extents in the different fractions. Another possibility is that HSF molecules are bound only loosely to some fraction and are easily liberated into solution during the fractionation procedures. This possibility seems to be the best explanation for the activity in the non-precipitated fractions, such as the cytoplasmic sap and M-I, obtained by centrifugation (Table 2). Nevertheless it seems significant that the specific activity of HSF in the outer membrane fraction was considerably higher than that in the spheroplasts or whole envelopes (crude membranes), as shown in Table 2.

Morse and Morse (1970) demonstrated filamentous structures, pili, on the cell wall and also presented evidence for a close association of the leukocytosis-promoting factor (LPF) and HSF with these structures. Sato et al. (1973, 1974) subsequently showed that LPF, HSF and hemagglutinin (HA) were carried by the same moiety, suggested to be the filamentous structures. More recently, however, LPF (=HSF) has clearly been separated from the pili, which are responsible for the HA activity (Morse and Morse, 1976). On the other hand, Parton and Wardlaw (1975) obtained two distinct bands on SDS-polyacrylamide gel-electrophoresis from envelopes of phase I organisms, but nor from those of phase IV organisms. These polypeptides, with MW of 30,000 and 28,000, disappeared when

the virulent phase I organisms were converted in culture to the non-virulent C-mode bacteria which lack HSF and the protective antigen (Wardlaw et al., 1976). There is now much suggestive, but not conclusive, evidence for the surface localization of HSF, and the present findings also indicate its high content, if not specific localization, in the outer membranes. Further investigations are in progress on the exact location of HSF and its relation with PIF.

The PIF activity measured in our assay systems was very low, $2–20 \times 10^3$ bacterial equivalents of extract being required to inactivate a single PMN cell; that is, as much as 10 pg of outer membrane protein per cell. This low activity seems insufficient for an effect of PIF in vivo. Experiments are in progress on the effect of PIF on PMN infiltrations in vivo and on Arthus reactions.

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