# ISOLATION AND PROPERTIES OF THE LEUKOCYTOSIS- AND LYMPHOCYTOSIS-PROMOTING FACTOR OF *BORDETELLA PERTUSSIS**

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In a previous report on the effects of the leukocytosis- and lymphocytosis-promoting factor (LPF)[1] of *Bordetella pertussis* on cyclic adenosine 3':5'-monophosphate (cAMP) metabolism, a method for isolation of highly active LPF was presented briefly (1). In the present communication, the results of further studies on the isolation and chemical, immunochemical, and biological properties of LPF are described. It has been found that a single moiety is responsible for the induction in mice of leukocytosis with both lymphocytosis and granulocytosis, histamine sensitization, and hypoglycemia and unresponsiveness to the hyperglycemic effects of epinephrine. In confirmation of our preliminary report (2), LPF has been found to be clearly distinct from the hemagglutinating pili of *B. pertussis*, the properties of which are also described.

## Materials and Methods

*B. pertussis Cultures.* Strain NIH 114 (3779B), which has been utilized in continuing studies in this laboratory was also employed in the present investigations. Lyophilized samples, obtained through the courtesy of Dr. Charles Manclark, Bureau of Biologics, Bethesda, Md., were reconstituted in sterile distilled $H_2O$ and plated on Bordet-Gengou medium (Clinical Sciences Inc., Whippany, N. J.). The 2-day growth at 36°C on one Petri dish was harvested in 10 ml of 2% casamino acids (technical grade; Difco Laboratories, Detroit, Mich.), and 1 ml of the suspension was inoculated into 100 ml of liquid medium in 1 liter Blake bottles. After 2 days of incubation at 36°C, 10 ml of the seed culture (approximately $5 \times 10^{10}$ organisms) was inoculated into 1 liter of liquid medium in Povitsky bottles which were then incubated at 35–36°C. In order to provide maximum surface area for aeration, all bottles were placed in the horizontal position during incubation. In the case of the 100 ml cultures, the depth of the medium was 1.0 cm and the surface area 218 $cm^2$; the values for the 1-liter cultures were 2.5 cm and 580 $cm^2$, respectively.

The liquid medium was that previously described (3) except for the omission of an anion exchange resin which was found to be unnecessary for either optimal growth, maintenance of the Phase I state, or maximal production of LPF. Culture density was assessed with the National Institutes of Health opacity standard, and the criteria utilized to determine whether the organisms were Phase I included: a homogenous population of small, gram-negative coccobacilli and

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; cAMP, cyclic adenosine 3':5'-monophosphate; CRBC, chicken erythrocytes; Con A, concanavalin A; HA, hemagglutinin; HI, hemagglutinin inhibition; HSF, histamine-sensitizing factor; LFP, leukocytosis- and lymphocytosis-promoting factor; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMNs, polymorphonuclear leukocytes; SDS, sodium dodecyl sulfate; WBC, leukocyte count.

the inability of the organisms to propagate on conventional media such as nutrient or trypticase soy agar.

*Animals.* Female mice of the Albany strain were used in earlier studies for assays of leukocytosis, but more recently 4- to 6-wk-old female CF-1 mice (Carworth Farms, Div. Charles River Breeding Laboratories, North Wilmington, Mass.) which respond in the same fashion with respect to leukocytosis, have been employed. CF-1 mice were also employed for determination of in vivo epinephrine responsiveness and histamine sensitization. 4- to 6-wk-old female HLA/SW (ICR) mice (Hilltop Laboratories, Scottsdale, Pa.) and CFW mice (Carworth Farms, Portage, Mich.) were also used in some experiments. "Nude" mice with a BALB/c background were bred and maintained in the Central Animal Service of the Downstate Medical Center.

*Biological Assays*

LEUKOCYTOSIS. Test material in a vol of 0.1 ml or 0.2 ml was injected intravenously via a lateral tail vein. At intervals, 5 $\mu$l or 10 $\mu$l of blood was obtained from the tail and diluted appropriately in 3% acetic acid containing crystal violet. The leukocytes were enumerated (leukocyte count [WBC]) in a hemacytometer. For routine assay of the LPF activity of a given preparation, groups of three to five mice were used and WBCs were performed 3 days after injection, a time previously shown to be optimal with respect to both total leukocytosis and lymphocytosis (3). Differential counts were performed on films stained with Wright-Giemsa.

HISTAMINE SENSITIZATION. After injection of *B. pertussis,* mice and rats which are normally resistant to the lethal effects of histamine become highly sensitive (4–7). Histamine-sensitizing factor (HSF) activity was assayed in pertussis-treated mice by injecting the equivalent of 1 mg of histamine-free base, as histamine dihydrochloride (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), intraperitoneally in 0.5 ml of isotonic saline and recording the number of deaths after 60 min.

BLOOD GLUCOSE AND EPINEPHRINE RESPONSIVENESS. Blood glucose was measured by the glucose oxidase method (Worthington Biochemical Corp., Freehold, N. J.). Fasting blood sugar was determined after removal of food for 5–6 h or overnight. Responsiveness to epinephrine was assayed by determining the blood glucose levels 30 min after the intraperitoneal injection of 5 $\mu$g of epinephrine (adrenalin HCl; Parke, Davis & Co., Detroit, Mich.) in 0.2 ml of phosphate-buffered saline (PBS).

*Hemagglutination and Hemagglutination Inhibition (HI).* Hemagglutination was routinely performed using chicken erythrocytes (CRBC). Test samples in a vol of 0.05 ml were serially diluted twofold in PBS in microtrays with U-shaped wells (Model IS-MRC-96, clear; Linbro Chemical Co., New Haven, Conn.) using the microtiter technique (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). Then 0.05 ml of an 0.5% suspension of washed CRBC in PBS was added, the trays gently shaken, and hemagglutination recorded after 60 min at room temperature.

HI was performed with sera heated at 56°C for 30 min. Because of the uniform finding of nonspecific HI in all normal rabbit and human sera tested, the sera were first absorbed with kaolin as described by Clarke and Casals (8). The adsorbed sera were then serially diluted twofold in a vol of 0.05 ml in borate-saline buffer (9) and then 0.05 ml containing four hemagglutinating units was added. After mixing, the trays were held at room temperature for 60 min, 0.05 ml of 0.5% CRBC was next added, and hemagglutination recorded after a further 60 min.

*Immunological Procedures.* Double-diffusion reactions were performed in 0.75% agarose in 0.1 M Tris-0.5 M NaCl, pH 9.0. Microimmunoelectrophoresis was usually carried out in barbital buffer, pH 8.2, ionic strength 0.04 (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) at 90 V for 90 min.

Antisera against *B. pertussis* and its products were prepared in rabbits which were repeatedly injected subcutaneously with 1.0 ml of killed whole culture. A useful schedule was to inject the animals three times a week for 3 wk and then wait 1–2 wk and repeat the series once or twice. Serum was obtained 7–10 days after the last injection. Antibody to purified LPF was prepared by homogenizing the LPF in complete Freund's adjuvant (Difco Laboratories) and injecting a total of 25 $\mu$g in multiple 0.2-ml depots. The injections were repeated in 3 wk and serum was obtained 10–21 days later. Hyperimmune pertussis human gamma globulin (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. and Cutter Laboratories, Berkeley, Calif.) was also employed.

*Chemical Determinations.* Protein was measured by the method of Lowry et al. (9) using

bovine serum albumin as the standard; nitrogen by the ninhydrin technique (10); carbohydrate by the phenol-sulfuric acid method (11); and total and bound lipid by the method of Marsh and Weinstein (12) after extraction of material into chloroform, chloroform:methanol, or heptane before and after alkaline hydrolysis. Elemental analyses were performed by Mr. T. Bella, The Rockefeller University, New York. Amino acid analyses were performed after hydrolysis of samples in vacuo in 6 N HCl at 110°C for 16 h, utilizing a Joelco model JL C-6AH analyzer (Joelco, Cranford, N. J.). Dr. Ronald F. Rieder, Downstate Medical Center, Brooklyn, N. Y., kindly assisted in the amino acid determinations.

*Polyacrylamide Gel Electrophoreses.* Analytical gel electrophoresis of undissociated or dissociated LPF was performed according to methods described by Maizel (13). Gels were stained with Coomassie blue and scanned with a soft laser scanner (Biomed Instruments, Inc., Chicago, Ill.). Analytical isoelectrofocusing was kindly performed by Dr. I. Parsa, Downstate Medical Center, Brooklyn, N. Y.

Elution of material in gels was performed in the following fashion. After electrophoresis, the cylindrical gel was cut longitudinally into quarters and one of the slices was stained for a brief period and destained electrolytically. The remaining segments were matched to localize the bands and then cut transversely in the appropriate areas. The cut portions were homogenized in 4 M urea-0.1 M $PO_4$-1 M NaCl, pH 6.4, and the gel then removed by centrifugation.

*Electron Microscopy.* Specimens on hydrophilic grids were negatively stained with 2% sodium dodecatungstosilicate or 1% ammonium molybdate. The preparations were examined in either a Siemens 1A (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) or Philips 300 (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.) electron microscope.

## Results

*Separation of LPF from Hemagglutinin (HA).* The basic methodology used to harvest and fractionate *B. pertussis* supernatant fluids has been described previously (1). Briefly, after 5 days of culture, 10% thiomersal in saline was added to achieve a final thiomersal concentration of 0.02%. After 24 h at 4°C, the bacterial cells were removed by centrifugation and the pH of the pooled supernatant fluid obtained from 6 to 10 liters of culture was adjusted to pH 6.4 with 1 N HCl. Solid $(NH_4)_2SO_4$ was added to 90% saturation while the pH was maintained by the addition of dilute $NH_4OH$. After 18 h at 4°C the precipitate was collected by filtration and resuspended in water. The $H_2O$ soluble fraction was discarded, and after washing the precipitate in 0.15 M NaCl, it was solubilized in 6 ml of 0.1 M Tris-0.5 M NaCl, pH 10 (Tris-NaCl buffer). Insoluble inactive material was removed by centrifugation and the soluble active fraction was layered on a discontinuous CsCl gradient. The gradient consisted of 6-ml portions of CsCl of densities 1.5, 1.3, 1.25, and 1.2 in Tris-NaCl buffer. The tubes were centrifuged in an SW-25 rotor at 50,000 *g* for 3.5 h. After centrifugation, the bulk of LPF, HSF, and HA was found in the 6 ml load vol which was then dialyzed against Tris-NaCl buffer.

Electron microscopy of this fraction (Fig. 1) revealed the presence of: filaments (pili) measuring 20–25 Å in diameter and of varying lengths (60–100 nm); heterogeneous round particles varying in diameter from 20–35 nm (14); and, in addition, smaller round particles were present. Morphologic heterogeneity was paralleled by immunological heterogeneity since immunodiffusion analysis revealed the presence of two major precipitin bands.

The fraction was then sieved through Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), in a 2.6 cm x 26 cm siliconized column. Elution was at 4°C with Tris-NaCl at a flow rate of 8–10 ml/h. 2-ml fractions were collected and the optical density at 280 nm measured. A representative

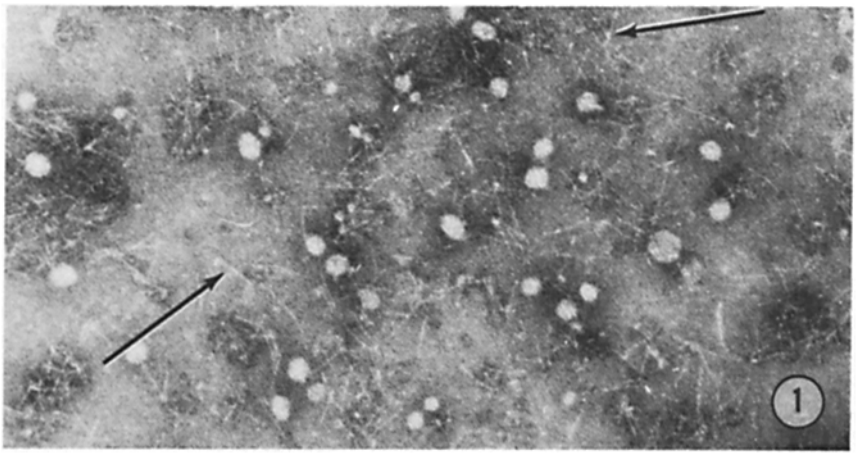


FIG. 1. Negatively stained fraction from a CsCl gradient containing LPF activity. Numerous pili (→), and large, round membranous particles are present. Also present are smaller round particles. × 140,000.

elution profile is shown in Fig. 2, together with the appearance of molecular weight marker substances (dextran blue dye, mol wt 2,000,000; phosphorylase A, mol wt 90,000; and bovine serum albumin (BSA), mol wt 67,000).

Three protein peaks were detected. Peak I was excluded from the Sephadex and coincided with the elution peak of the excluded dextran blue dye. Peak II was eluted after phosphorylase A and in the same region as BSA. The third peak had neither biological nor immunological reactivity and will not be considered further. Peaks I and II were each concentrated on an Amicon apparatus using a PM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and then tested for HA and LPF activity.

As can be seen in Table I, the excluded peak I contained a highly potent HA but was devoid of LPF activity. In contrast, peak II was a potent inducer of leukocytosis but had only minimal hemagglutinating capacity. The increases in specific activity over the starting culture supernatant fluid was 40- to 50-fold for the HA fraction (peak I) and 25- to 40-fold for the LPF fraction (peak II).

*Properties of HA.* General properties of the HA were first examined using culture supernatant fluids as the source of crude HA. It was found that erythrocytes from a number of species other than chickens were agglutinated. These included erythrocytes of man (O negative), rabbit, sheep, guinea pig, and rat. Hemagglutination was not inhibited by mannose or α-methylmannoside (15). Treatment of erythrocytes with crude *Vibrio cholerae* neuraminidase did not cause a reduction in the titer of pertussis HA. Thus, the receptors for the HA were not neuraminidase sensitive. The HA was sensitive to heat with over 90% of the activity destroyed in 30 min at 56°C and was inactivated by the proteolytic enzymes trypsin and pronase. However, it was not inactivated by either 0.1% formalin or 5.0% phenol after exposure for 48 h at 4°C.

HA-inhibition tests were performed using the G-150 peak I HA fraction and it was found that antisera prepared in rabbits against whole cultures of *B. pertussis* had HI titers ranging from 1/40–1/160. It was of note that sera from 12

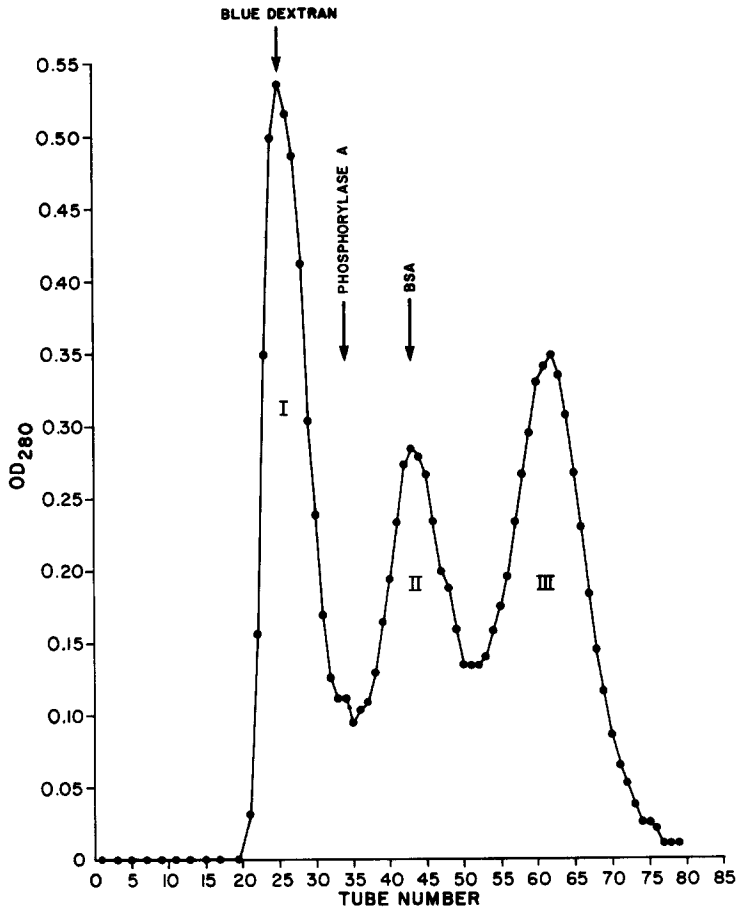



FIG. 2. Sephadex G-150 column chromatograph of an LPF-containing fraction obtained from a CsCl gradient.

TABLE I
*HA and LPF Activity of Sephadex G-150 Peaks*

| Peak | 1 HA unit* | WBC × $10^{-3}/mm^3$‡ |
|---|---|---|
| | *μg* | |
| I | 0.03 | 23.7 |
| II | 0.5 | 182.0 |

* Minimal quantity producing complete hemagglutination.
‡ 3 days after the intravenous injection of 1 μg (normal WBC, 20.5 × $10^{-3}/mm^3$). Mean value in four mice.

immunized children ranging in age from 16 mo to 6 yr had even weaker HI titers (less than 1/10).

The ultrastructural components of the HA fraction are illustrated in Fig. 3. The predominant structures are the previously noted pili and membrane-like components but the smaller particles were absent. The structured elements were partially resolved by ultracentrifugation. HA at a concentration of 500 μg

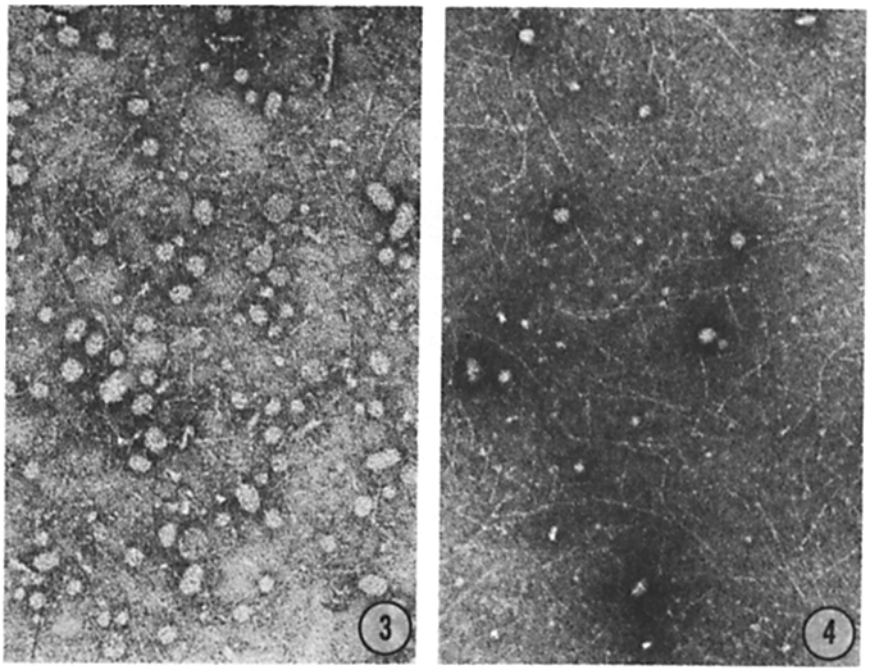



FIG. 3. Electron microscope appearance of peak I from a Sephadex G-150 column which contained HA activity. Numerous pili and large membranous particles are present. × 140,000.

FIG. 4. Supernate of the HA fraction after centrifugation. Pili are now the predominant structures. × 140,000.

of protein/ml was centrifuged at 30,000 RPM for 2.5 h in an SW 39 rotor. The supernatant fluid was removed and recentrifuged under the same conditions. The pellets were pooled and resuspended to initial volume and the preparations analyzed for HA activity, ultrastructural appearance, and immunoprecipitin reactions. The specific activity for HA in both the supernate and pellet was identical; 0.04 $\mu$g produced complete hemagglutination.

Electron microscopic examination of the supernate revealed mainly pili (Fig. 4), whereas the pellet contained both pili and membranous particles. Paralleling this observation was the finding that both the starting material and the pellet contained two immunologically reactive bands, whereas the supernatant fraction yielded only one prominent band. The combined immunological and ultrastructural findings therefore suggested that the HA activity of *B. pertussis* resided in the pili.

*Properties of LPF.* When concentrated peak II, at a concentration of 500 $\mu$g/ml, was tested against a battery of different whole culture antisera most preparations showed only a single immunoprecipitin band. Occasionally preparations were found to contain a second immunologically reactive component. LPF was readily removed from this component by dialyzing the preparation against 400 volumes of PBS at 4°C for 5 h. The precipitate which formed was dissolved in Tris-NaCl buffer and contained only LPF, whereas the PBS supernate contained both components. Highly purified LPF with only a trace of the

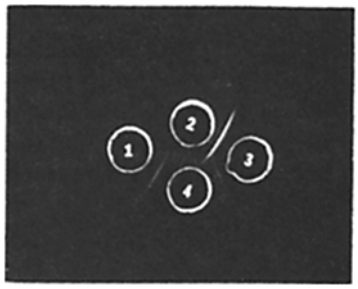



FIG. 5. Well 1, contains anti-LPF antiserum; well 2, the CsCl load fraction before chromatography on Sephadex G-150; well 3, whole antipertussis antiserum; and well 4, purified LPF. Note that the pertussis antiserum recognizes only one immunologically reactive component in LPF, and that anti-LPF antiserum reacts with only a single component in the crude material.

second component could be prepared by dialyzing the PBS supernatant fluid against large volumes of $H_2O$ for 18 h and harvesting the precipitate. The water-soluble material had no LPF activity.

LPF preparations tended both to aggregate and to lose activity. When 4 M urea-0.1 M $PO_4$-1 M NaCl with an apparent pH of 6.4 (16) was utilized, aggregation was minimized and LPF solutions in this medium retained activity when stored at concentrations of 100–700 $\mu$g/ml for periods of 6–10 wk; in contrast, LPF activity was rapidly lost in 2.5 M guanidine in the same buffer.

*Homogeneity of LPF.* Homogeneity of LPF prepared in this fashion was analyzed by immunological, physical, and electron microscopical techniques.

In double-diffusion analyses, the isolated LPF gave a single immunological precipitin band when reacted over a wide range of concentrations with heterospecific antipertussis antibody. Similarly, at various dilutions of antiserum and a constant antigen amount, only a single immunoprecipitin band was detected.

Antisera raised to the isolated LPF also showed a single band on Ouchterlony analysis against LPF and recognized only a single immunoreactive substance in crude concentrated pertussis fractions in which other components of the organism as well as LPF were present (Fig. 5). Human hyperimmune pertussis gamma globulin also produced a single band in double-diffusion reactions with LPF, while pooled normal human gamma globulin caused only a very weak reaction (Fig. 6). Normal rabbit serum did not precipitate LPF. Immunoelectrophoretic analyses also showed that when LPF was electrophoresed and then heterospecific pertussis antisera or LPF antisera added, a single immunologic component was detected (Fig. 7). In some preparations a second arc which fused with the main precipitin arc was present. However, under a variety of different conditions the arcs never completely crossed and always fused, indicating a reaction of partial identity. These findings suggest that some degradation of the LPF molecule may have taken place *(vide infra)*.

A number of different acrylamide gel concentrations and buffers were utilized in attempts to analyze undissociated LPF. Irrespective of gel concentrations, only trace amounts of material entered when the buffer pH was neutral or alkaline. The most useful system proved to be that composed of 5% gels and the pH 4.5 buffer described by Reisfeld et al. (17) but without the stacking gel.

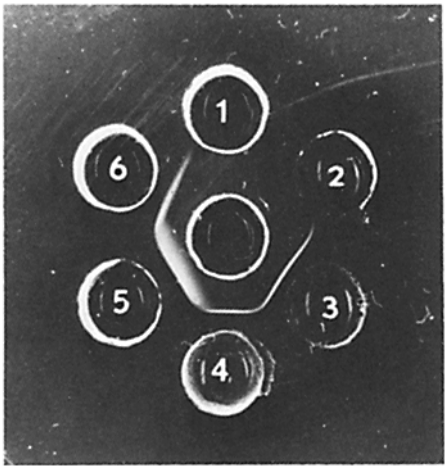



FIG. 6. The center well contains purified LPF. Well 1 contains normal rabbit serum and well 2 contains pooled normal human gamma globulin. Different batches of human hyperimmune pertussis gamma globulin are present in wells 3 and 4, while anti-LPF antiserum is in well 5 and antipertussis antiserum is in well 6.

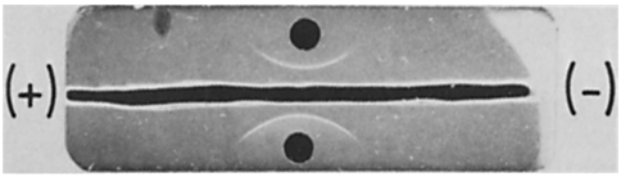



FIG. 7. The top and bottom wells contain two different preparations of LPF which were subject to electrophoresis before the addition of antipertussis antiserum to the center trough.

Under these conditions, the vast bulk of the material was present as a single band with one or two shoulders toward the cathodal end. It was believed that the shoulders represented degradative products of LPF since when stored LPF, which had lost activity, was rerun, the peak shifted toward the cathode without apparent loss of total protein (Fig. 8).

Isoelectric focussing analysis of LPF revealed four minor components together with a major peak and its shoulder which had isoelectric points of approximately 9.4–9.6. These last, on the basis of electrophoretic properties, most likely represented the major peak with its shoulder seen in acrylamide gels.

Immunological reactivity of undissociated material in the analytical polyacrylamide gels was studied by electrophoresing LPF, cutting the gel lengthwise in half, and staining one half. The other half was embedded in agarose. After, 30-min troughs were cut in the agarose parallel to and 0.5 cm from the gel. One trough was filled with normal rabbit serum and the other with antipertussis antiserum. A single immunoprecipitin band coinciding with the major peak was found.

Together these observations indicated that the LPF preparation consisted of a major fraction which accounted for at least 70–80% of the protein present,

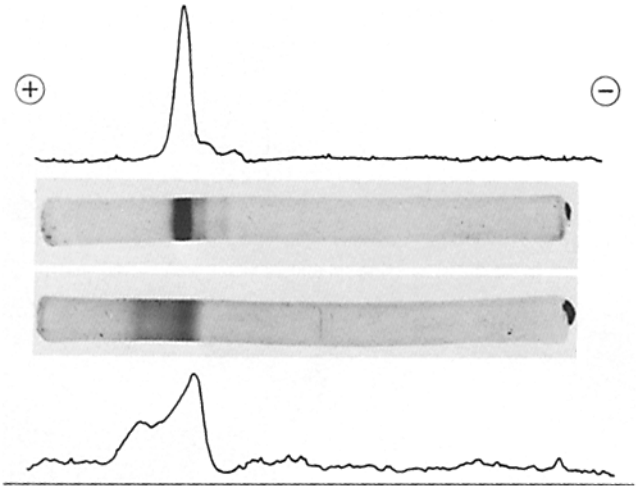
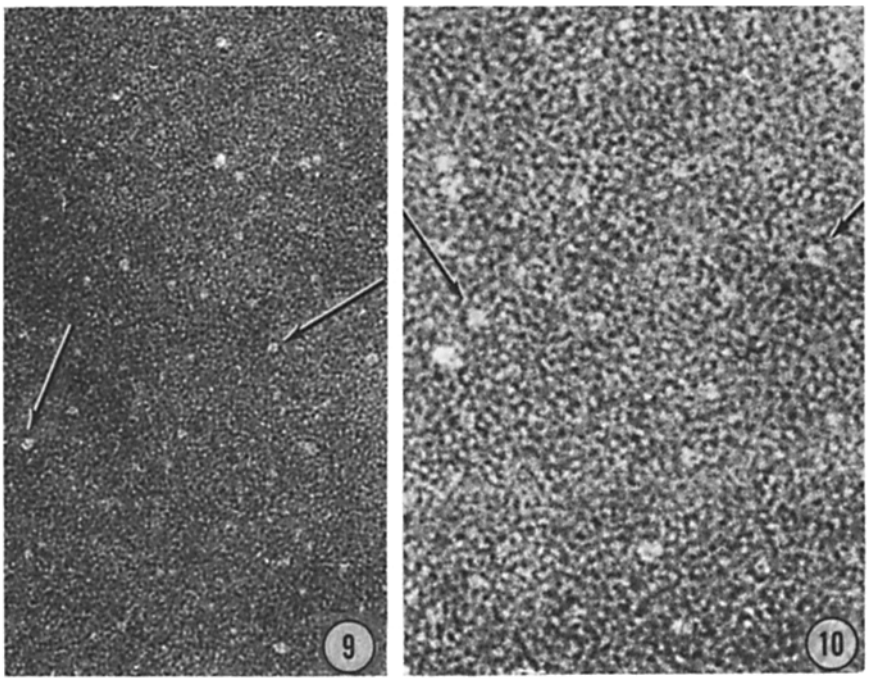



FIG. 8. Polyacrylamide gel electrophoretic protein pattern of LPF. Upper two panels: densitometric scan and stained gel of active LPF. Lower two panels: stained gel and densitometric scan of the same LPF which had become inactive on storage.

depending upon the preparation, and that there was an immunoreactive substance corresponding to this peak. There was also evidence that the heterogeneity observed was related, at least in part, to the presence of degraded or denatured LPF.

As illustrated in Figs. 9 and 10 the purified preparations of LPF contained round particles, usually ringshaped, which measured 75–80 Å in diameter. Although the ring or doughnut structure predominated, occasional round particles were noted which lacked the central hole. Each ring was composed of four to five round subunits measuring 28 Å in diameter. Occasionally the subunits gave the appearance of projecting spikes. The preparations in urea, also found to be biologically active, were the most helpful in providing particles of smallest uniform size. Frequently, preparations in borate or Tris buffer, especially in high concentration, contained large aggregates of the 75–80 Å ring structures so that the smallest uniform unit was hard to ascertain.

A number of different LPF preparations were analyzed by polyacrylamide gel electrophoresis in 10% running gels after dissociation by boiling for 1 min in 4 M urea, 1% sodium dodecyl sulfate (SDS), and 0.2 M 2-mercaptoethanol (12). In each instance four polypeptides were detected (Fig. 11). Utilizing a standard solution containing bovine serum albumin, mol wt 67,000; ovalbumin, mol wt 45,000; chymotrypsinogen, mol wt 25,000; and RNase A, mol wt 13,700, the mol wt of the polypeptides were determined to be 23,500, 19,300, 17,400, and 13,400. The minimal mean mol wt of LPF as determined by this method, assuming that the molecule is made up of one each of the polypeptides, is 73,600, which is in reasonably good agreement with that estimated by molecular sieving on Sephadex G-150. However, there is preliminary evidence that the lowest molecular weight component is present in twice the molar proportions of the other polypeptides, which, if confirmed, would place the minimum mol wt by this technique at approximately 87,000.




FIGS. 9 and 10. Electron micrograph of purified LPF. Fig. 9, × 400,000. Fig. 10, × 200,000. Arrows denote molecules corresponding to LPF activity. Note the absence of pili.

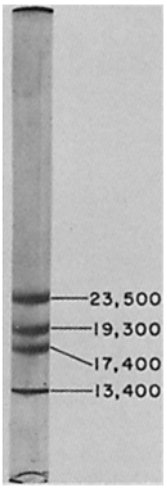



FIG. 11. Polyacrylamide gel electrophoresis of LPF dissociated in SDS and 2-mercaptoethanol.

Since the amino acid sequences of the subunits of LPF are unknown it is difficult to be sure how the packing of the amino acids takes place in the individual subunits. The finding of four bands on SDS gel, with the possibility that the smallest band is present in twice the concentration of the other three, is in agreement with the electron microscope picture of four to five small subunits.

TABLE II
*Amino Acid Composition of LPF*

| Amino Acid | μM per 100 μM | Amino Acid | μM per 100 μM |
|---|---|---|---|
| Lysine | 5.0 | Glycine | 10.4 |
| Histidine | 1.3 | Alanine | 12.2 |
| Arginine | 6.2 | ½ Cystine | 0.8 |
| Aspartic acid | 8.1 | Valine | 4.6 |
| Threonine | 6.5 | Methionine | 2.4 |
| Serine | 6.7 | Isoleucine | 3.2 |
| Glutamic acid | 9.5 | Leucine | 8.1 |
| Proline | 7.0 | Tyrosine | 4.5 |
| | | Phenylalanine | 3.5 |

*Chemical Analysis of LPF.* Carbohydrate was not detected at the 1% limit of the analysis utilized, and lipid was not found either before or after alkaline hydrolysis at a limit of less than 0.5%. The UV absorption spectrum of an 800 μg/ml solution of LPF revealed a peak at 280 nm but not at 260 nm, indicating the absence of nucleic acid. A representative amino acid analysis is shown in Table II. No amino sugars were detected. The elemental analyses were: N, 14.5%; C, 47.9%; and H, 6.5%.

*Biological Properties of Purified LPF: Leukocytosis and Lymphocytosis.* As little as 0.02 μg of LPF protein when injected intravenously resulted in a significant leukocytosis 3 days after inoculation (Table III). In Fig. 12 the time-course of the response to 0.5 μg of LPF is depicted in respect to the total WBC and the number of circulating lymphocytes and polymorphonuclear leukocytes (PMNs). It can be seen that as early as 6 h after injection of LPF, there was an increase in all three parameters which gradually rose to a peak between the 3rd and 5th day after injection. By the 7th day after inoculation, the cell count values began to decrease, but normal values were not reached until the 3rd or 4th wk.

When higher doses of LPF were used it was found that with doses of 0.5, 1.0, or 2.0 μg there was little difference in the total lymphocyte counts 3 days after injection, although the total WBC and PMN counts were higher in the groups receiving 1.0 or 2.0 μg. When 4 μg of LPF was given, however, both the lymphocyte and PMN responses were greater. During the first 3 days there was no mortality and all groups showed weight gain, although this was slightly less in the groups receiving the two highest doses. By the 7th day after injection, two of four and one of four mice which had received 4 μg and 2.0 μg of LPF, respectively, had died. There was marked weight loss in the surviving animals in these two groups. Moreover, in contrast to the observations in animals given lower doses of LPF the number of circulating lymphocytes and PMNs had markedly increased. By the 21st day, all of the mice that had received 4 μg of LPF and three of the four which had received 2 μg were dead. There was no mortality in the groups receiving 1 [g or less and the changes in weight were equal to those in the control mice. Thus at high doses of LPF, in addition to toxicity, there was a shift in the time, as well as in the magnitude of the peak response, rather than simply a heightened response at the earlier time period.

The question of the kinds of lymphocytes, T cells and/or B cells, responding to

TABLE III
*Leukocyte Response of Mice to LPF*

| LPF | WBC × $10^{-3}$/mm$^3$ |
|---|---|
| $\mu g$ | |
| 0.5 | 159.2 ± 10.6 |
| 0.1 | 85.4 ± 8.3 |
| 0.02 | 35.2 ± 5.2 |
| None | 15.7 ± 3.4 |

Groups of four CF-1 mice were injected intravenously with LPF and leukocyte counts were performed 3 days later.

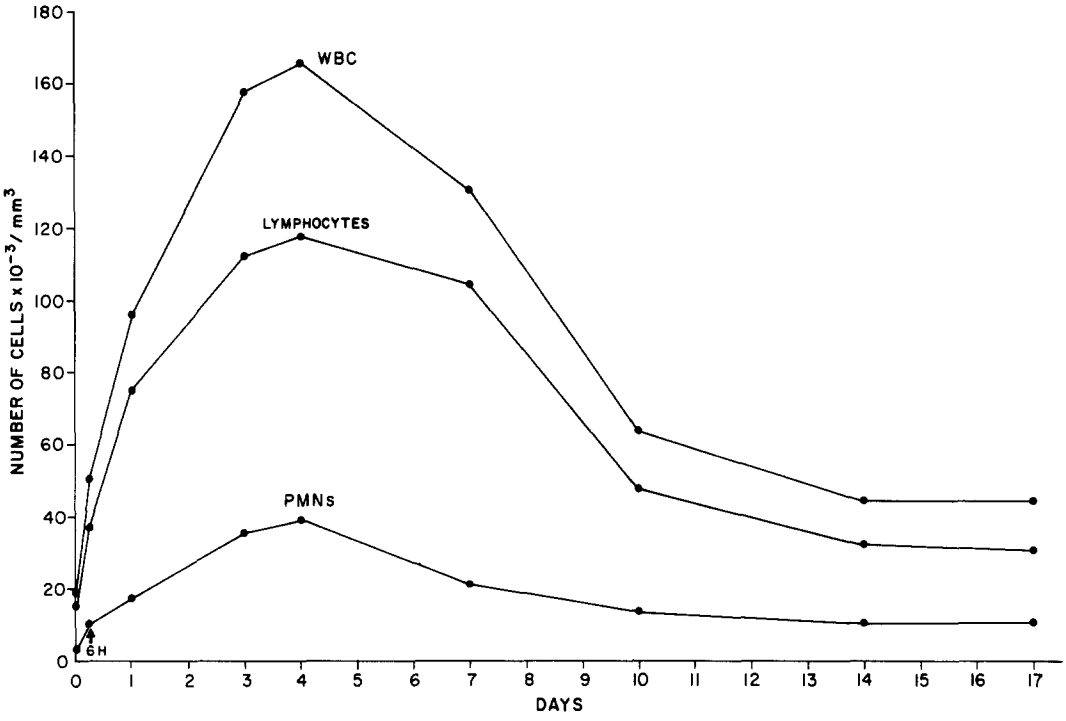



FIG. 12. The leukocyte response of mice to the intravenous injection of 0.5 $\mu$g of LPF.

LPF was investigated. In preliminary experiments performed in cooperation with Doctors C. Bianco and V. Nussenzweig, New York University School of Medicine, New York, it was found that B lymphocytes as well as T lymphocytes were both increased in the blood and although T cells were still the preponderant cell, the relative increase in B lymphocytes was greater. This change was accompanied by a relatively greater depletion of B cells in the spleen.

In order to further study the effect of LPF on B lymphocytes, congenitally athymic "nude" mice, which lack T cells, were utilized. It can be seen in Table IV that homozygous nu/nu mice responded with a leukocytosis which averaged 5.4 times base line values and that the number of circulating lymphocytes and PMNs increased 4.9- and 7.7-fold, respectively. The overall response to this dose

TABLE IV
*Effect of LPF on the Blood Leukocytes of Nude Mice*

| Mouse Strain | Day | WBC* | No. of lymphocytes* | No. of PMNs* |
|---|---|---|---|---|
| BALB/c | 0 | 8.9 ± 2.7 | 6.7 ± 1.8 | 1.8 ± 0.8 |
| | 1 | 26.6 ± 6.4 | 20.8 ± 5.5 | 4.6 ± 1.4 |
| | 3 | 63.3 ± 3.3 | 49.9 ± 6.3 | 9.7 ± 3.6 |
| | 7 | 47.3 ± 5.3 | 36.9 ± 1.8 | 8.0 ± 3.9 |
| nu/BALB | 0 | 13.8 ± 2.1 | 11.4 ± 2.2 | 2.0 ± 0.4 |
| | 1 | 31.8 ± 5.0 | 26.2 ± 3.1 | 4.3 ± 1.4 |
| | 3 | 39.7 ± 7.0 | 33.0 ± 6.2 | 5.7 ± 1.9 |
| | 7 | 28.3 ± 5.4 | 22.7 ± 3.9 | 4.0 ± 1.3 |
| nu/nu | 0 | 7.9 ± 0.5 | 6.0 ± 1.5 | 1.4 ± 1.1 |
| | 1 | 21.7 ± 8.7 | 14.5 ± 5.1 | 6.0 ± 3.7 |
| | 3 | 42.6 ± 3.4 | 29.5 ± 1.4 | 10.8 ± 4.8 |
| | 7 | 35.1 ± 7.6 | 27.2 ± 7.6 | 6.2 ± 2.6 |

Mice in groups of four were injected intraperitoneally with 0.5 μg of LPF and leukocyte counts performed at intervals.
* Number of circulating cells × $10^{-3}$/mm$^3$ ± 1 SD.

of LPF was less than usually observed because the material was injected intraperitoneally, a less effective route than the intravenous one.

*Histamine Sensitization.* Several different strains of mice were tested for their ability to be sensitized to the lethal effect of 1 mg of histamine by LPF. A dose of 10 mg of histamine was insufficient to kill normal mice of any of these strains but the injection of 0.5 μg of LPF 3 days before challenge markedly increased their sensitivity and all were killed by 1 mg of histamine. The dose of LPF required to induce sensitization to this dose of histamine in 50% of challenge mice ($SD_{50}$) varied with the mouse strain being tested as shown in Table V; the highest $SD_{50}$ of LPF was 0.33 μg for Albany mice and the lowest was 0.01 μg for CF-1 mice. The differences in capacity to be sensitized to histamine by LPF did not lie in intrinsic susceptibility of untreated mice to the amine. Thus while all of the mouse strains tolerated a dose of 10 mg of histamine, 20 mg was lethal for 100% of Albany strain mice for which the $SD_{50}$ of LPF was the highest, as well as for CF-1 mice for which the $SD_{50}$ was the lowest, but this dose was not lethal for normal HLA/SW mice which were also readily sensitized by LPF.

The optimal time of sensitization of CF-1 mice was 3–5 days after injection of LPF. At 2 wk, sensitization was diminished and at 4 wk none of five mice which had received 0.5 μg of LPF were killed by 1 mg of histamine.

*Epinephrine Refractory Hypoglycemia.* It is known that injection of *B. pertussis* cells into experimental animals results in hypoglycemia and a decreased responsiveness to epinephrine as measured by a lack of elevation of blood sugar after injection of the catecholamine (18–20). Table VI presents the results of a representative experiment in which various doses of LPF were injected intravenously into CF-1 mice. 3 days later the blood sugar was determined in the fasting state and 30 min after the intraperitoneal injection of 5 μg of epinephrine. It can be seen that there was no hyperglycemic response to

TABLE V
*50 Percent Sensitizing Dose of LPF for Histamine Lethality in Various Strains of Mice*

| | Mouse strain | | | |
|---|---|---|---|---|
| | Albany | CF-W | HLA/SW | CF-1 |
| $SD_{50}$ ($\mu$g)* | 0.33 | 0.05 | 0.03 | 0.01 |

3 days after the intravenous injection of various amounts of LPF, groups of five mice were injected intraperitoneally with an amount of histamine diHCl equivalent to 1 mg of histamine base. Deaths were recorded 60 min later.

* Dose of LPF required to sensitize 50% of the mice to the lethal effect of 1 mg of histamine.

TABLE VI
*The Effect of LPF on Epinephrine Responsiveness*

| LPF Dose | Blood glucose (mg %) | |
|---|---|---|
| | Fasting | 30 min after epinephrine |
| $\mu$g | | |
| 1.0 | 96.8 ± 5.8 | 67.0 ± 19.4 |
| 0.5 | 83.8 ± 7.4 | 87.5 ± 11.2 |
| 0.1 | 82.8 ± 5.0 | 92.0 ± 7.8 |
| 0.02 | 89.5 ± 4.8 | 109.8 ± 7.2 |
| None | 102.5 ± 10.9 | 216.5 ± 27.8 |

3 days after the intravenous inoculation of LPF, the fasting blood glucose was determined in groups of four CF-1 mice. Then, 5 $\mu$g of epinephrine was injected intraperitoneally and the blood glucose was determined 30 min later. Values are the means ± one standard deviation.

epinephrine in mice which had received between 0.1 and 1.0 $\mu$g of LPF and only a minimal response, compared to that found in control mice, in those which had received 0.02 $\mu$g of LPF. In the unresponsive mice a depression of the fasting blood glucose was also observed. When 0.5 $\mu$g of LPF was injected intravenously, the lack of responsiveness to epinephrine persisted unaltered at 2 wk and was still less than 50% of that in control animals at 1 mo.

It thus appeared that a single moiety had both LPF and HSF activity, and, in addition, was able to block the increment in blood glucose which normally follows the administration of epinephrine. Further evidence for the unity of these reactions was obtained when it was found that all three activities were present in the eluate of the major band appearing on polyacrylamide gel electrophoresis and not in any other fraction. In addition, all three reactions were blocked both by prior incubation of LPF with monospecific antiserum prepared against the isolated compound (Table VII), and by in vivo injection of the antiserum.

## Discussion

Phase I cells of *B. pertussis* and their products induce a number of diverse effects in experimental animals (reviewed in references 5, 7, 21, 22). Among

TABLE VII
*The Effect of Antiserum on the Ability of LPF to Induce Leukocytosis, Histamine Sensitization, and Fasting Hypoglycemia and Epinephrine Unresponsiveness*

| Serum | WBC × $10^{-3}$/mm$^3$ | Histamine deaths/ total | Blood glucose (mg %) | |
|---|---|---|---|---|
| | | | Fasting | After epinephrine |
| None | 72.0 ± 15.4 | 4/4 | 103 ± 1.48 | 112 ± 16.4 |
| Normal | 96.5 ± 14.8 | 4/4 | 99 ± 5.4 | 99 ± 8.9 |
| Anti-LPF | 18.5 ± 14.5 | 0/4 | 133 ± 5.9 | 191 ± 42.3 |
| Control | 13.1 ± 3.8 | 0/4 | 146 ± 14.7 | 214 ± 21.9 |

LPF was incubated for 18 h at 4°C with normal rabbit serum, rabbit anti-LPF serum, or buffer alone. After centrifugation, 0.1 ml of the supernatant fluid was injected intravenously into CF-1 mice. 3 days later leukocyte counts, sensitization to the lethal effects of 1 mg of histamine, and the fasting blood glucose and blood glucose response to 5 μg of epinephrine were determined. The dose of LPF injected was greater than five times that required to produce leukocytosis and unresponsiveness to epinephrine, and 40 $SD_{50}$s with respect to histamine sensitization.

these effects are: lethality and dermonecrosis caused by a heat-labile component(s); responses, such as fever, characteristic of the lipopolysaccharide endotoxin moiety of the cell walls of gram-negative organisms; sensitization to the lethal effects of the pharmacologic agents histamine and serotonin, as well as to such nonspecific stresses as cold, endotoxic shock, and peptone shock; enhancement of anaphylaxis; immunopotentiation with respect to conventional antibody, homocytotropic antibody, and delayed hypersensitivity; hypoglycemia and decreased hyperglycemic response to epinephrine; and leukocytosis with a predominating lymphocytosis. In addition, it is clear that whole cells and/or crude extracts of Phase I organisms induce protective immunity against pertussis, both in animals and man.

Phenomena exhibited in vitro are hemolysis, hemagglutination, and inhibition of isoproterenol and prostaglandin $E_1$ stimulation of cAMP accumulation in human lymphocytes (1). Little definitive information exists on the nature and the interrelationships of the components responsible for these reactions with the notable exception of the endotoxin complex which seems to have the same general structural and biological activities of other compounds of this type (23–25).

Over the last several years we have been engaged in studies designed to isolate and characterize the factor that induces leukocytosis with a predominant lymphocytosis, LPF, and to determine whether LPF has other biological activities known to be induced by *B. pertussis*. In initial studies, using supernatant fluids obtained from cultures grown in a dializable medium, it was found that LPF activity was concentrated in a cesium chloride gradient fraction which contained as its dominant element filaments measuring 20 Å in diameter and 40–70 μm in length (14). This same fraction had marked histamine-sensitizing activity and was hemagglutinating. When such CsCl fractions were passed through Sephadex G-150, the filaments and hemagglutinating properties were excluded from the gel and clearly separated from LPF activity as well as from HSF (12). The HA found in the void volume was markedly sensitive to heat and to proteolytic enzymes. Differential centrifugation strongly suggested that the hemagglutinating properties did reside in the filamentous particles. These

results are consistent with the findings of Sato and his colleagues (26–28) but they also suggested that the filaments were identical with LPF and HSF, an observation that is not confirmed by the present studies. They also proposed that the filaments can induce protection against experimental pertussis, an observation which preliminary results in our laboratory tend to verify.

The filaments are found on the surface of the organisms early in growth in both liquid and solid culture and then in the extracellular environment (14, 28). The same is true for the HA (29). Although in our initial studies the terms "pili" and "fimbriae" were avoided because of the narrower diameter of the filaments (20 Å) compared with pili of other organisms (30) their ultrastructure, localization, protein nature, and hemagglutinating ability would seem to make the terms appropriate.

The LPF, in contrast to HA, entered Sephadex G-150 and was eluted in the same region as BSA. LPF contained 14.5% nitrogen and appears to be protein in nature. In this regard, it is of note that despite exhaustive studies, lipid was not found. This is in striking contrast to the results of Lehrer and co-workers who reported the isolation of a homogeneous product from cells of *B. pertussis* which had LPF as well as HSF activity and which contained greater than 50% lipid (16). In their filament-rich preparations with LPF activity Sato and Arai (26) also reported the presence of 17.5% lipid, and Munoz and Hestekin (31) found 28.4% lipid in partially purified HSF, but in these cases the products were clearly heterogenous.

Lehrer et al. (16) used whole cells as starting material, and it is possible that their preparations were contaminated with lipopolysaccharide endotoxin. It is also possible that when LPF is attached to the cell it is bound to lipid and that spontaneous release of the material involves dissociation of a protein-lipid bond. Another difference in the chemical nature of the two preparations is that Lehrer et al. (16) could not detect any sulfur-containing amino acids, whereas the LPF isolated in this laboratory contained small amounts of both cysteine and methionine.

LPF was found to consist of four polypeptide chains and the mol wt as determined by acrylamide gel electrophoresis was approximately 72,000, whereas a value of approximately 67,000 was found using Sephadex chromatography. However, if the finding of 2 mol of the lowest molecular weight peptide for each one of the others is confirmed then the minimal mol wt as determined by gel analysis would be 86,000. It is of interest that the electron microscopical appearance of LPF is commensurate with a structure composed of four or five subunits.

When as little as 0.02 $\mu$g of LPF was injected intravenously into mice, a significant leukocytosis was found 3 days later. Optimal responses at this time period were obtained when doses in the range of 0.1–1.0 $\mu$g were used and no toxicity was seen; neither lethality nor weight loss in excess of control mice was observed. At doses of 2–4 $\mu$g, the peak response was shifted to a later time period and both weight loss and lethality occurred.

When 0.5 $\mu$g of LPF was inoculated, a leukocytosis was observed as early as 6 h later and both the circulating lymphocytes and granulocytes were increased in number. The leukocytosis increased to a peak at days 3–5. At day 7 the number

of circulating leukocytes had decreased and during the next 2–3 wk normal values were achieved. The changes in total leukocyte count and in the number of lymphocytes and PMNs were essentially parallel.

It was striking that the early, transient, polymorphonuclear leukocytosis previously noted 6 h after injection of crude supernatant fluids was no longer found (3). It is probable that this reaction was due to contaminating endotoxin, a thesis supported by the morphologic finding of material in crude supernatant fluids which appeared to be derived from extrusions of the cell wall (14). The isolated LPF was free of lipopolysaccharide endotoxin by chemical and electron microscopical criteria. Moreover, anti-LPF antiserum did not react with pertussis endotoxin prepared by the phenol-water technique.

LPF increased the number of both circulating B cells and T cells. The increment in circulating lymphocytes in nude mice, which lack T cells, was more striking than found in the studies of Wortis (32) and Finger et al. (33). These workers used whole *B. pertussis* cells and there may be differences in the responsiveness of nude mice to intact cells and to soluble products. In addition to thymus aplasia of the nude mice employed in these studies, confirmatory evidence of their lack of T cells was derived from the unresponsiveness of either their spleen cells or lymph node cells to T-cell mitogens such as phytohemagglutinin (PHA) and concanavalin A (Con A), whereas these cells did undergo a marked proliferative response to endotoxin, a B-cell mitogen. It was also clear that LPF did not induce maturation of precursor T cells into functional T cells, since the cells at the height of the reaction also did not respond to PHA, although they still responded to endotoxin.

In addition to its marked capacity to induce leukocytosis and lymphocytosis, LPF was also extremely potent in sensitizing mice to the lethal effects of histamine. Depending upon the mouse strain used, the $SD_{50}$ for 1 mg of histamine base ranged from 0.01 to 0.33 $\mu$g of LPF. As in the case of leukocytosis, peak sensitization was observed 3–5 days after injection of LPF, and the responsiveness decreased over the next several weeks.

The LPF preparations were also exceedingly active in inhibiting the increment in blood glucose which normally follows the injection of 5 $\mu$g of epinephrine into fasting animals. Although complete end-point titrations were not performed, when 0.02 $\mu$g of LPF was injected intravenously there was only minimal responsiveness to epinephrine 3 days later. In addition, fasting hypoglycemia was found after administration of optimal doses of LPF.

Certain general findings, such as the time of peak response and the concordance of relative activities in a given preparation had suggested that the three biological activities – leukocytosis and lymphocytosis, histamine sensitization, and hypoglycemia and refractoriness to epinephrine – were produced by a single product of *B. pertussis*.

Critical evidence in favor of a single responsible substance was obtained in two different ways. Firstly, it was found that all three activities of LPF were abolished by an antiserum which recognized only a single immunoreactive substance. Most convincing, was that the eluate of a single band on gel electrophoresis produced the three phenomena.

It seems probable that LPF is also responsible for the elicitation of enhanced

sensitivity to serotonin and nonspecific stress which usually accompany histamine sensitization (5, 7). Lehrer et al. (16, 34), as well as Tada et al. (35) have also reported that preparations rich in LPF enhance the production of reaginic, but not of conventional, antibody to ovalbumin. We have not as yet examined the ability of purified LPF to increase the homocytotropic antibody response.

Recently, we have found that LPF has still another biological activity; it is an exceedingly effective mitogen for murine T lymphocytes (36) as well as for nonimmune human lymphocytes (J. H. Morse, A. S. Kong, and S. I. Morse, unpublished observations).

With such a diversity of activities it will be of great importance to determine precisely the mechanism(s) of action of LPF and particularly whether a single common reaction induces the range of observed biological effects. From the evidence at hand, further investigation of the relationship between LPF and cyclic nucleotides is certainly warranted. It is known that both histamine sensitization (37) and refractoriness to the hyperglycemic effect of epinephrine (38) can be produced by beta adrenergic antagonists, and in turn many of the effects of beta adrenergic agonists are mediated by cAMP. In addition LPF blocks the intracellular accumulation of cAMP which is normally stimulated by beta adrenergic agents such as isoproterenol (1). However, the reaction is more complex than that of beta adrenergic blockade, since the ability of prostaglandin $E_1$ to increase cAMP is also inhibited. It is thus possible that LPF has a direct effect on adenylate cyclase, the activity of which appears to be decreased in the spleens of pertussis-treated mice (39). An alternative hypothesis is that cGMP metabolism is affected, with a consequent reciprocal effect on cAMP. It would be of great interest if a role for cyclic nucleotides in the control of lymphocyte recirculation is revealed in the ongoing studies of the mechanism of LPF action.

## Summary

The leukocytosis- and lymphocytosis-promoting factor (LPF) of *Bordetella pertussis* has been isolated to near homogeneity by physical, chemical, and electron microscopical criteria. LPF contains 14.5% nitrogen and is lipid and carbohydrate free. It is apparently composed of four polypeptide subunits.

LPF caused leukocytosis and lymphocytosis in "nude" as well as in normal mice. In addition, purified LPF also induced histamine sensitization and hypoglycemia and refractoriness to the hyperglycemic effect of epinephrine. A monospecific LPF antiserum blocked these reactions as well as leukocytosis and lymphocytosis. LPF is clearly distinct from the hemagglutinating pili of *B. pertussis*.

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