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Leukocytosis-Promoting Factor of *Bordetella pertussis*

II. Biological Properties

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The leukocytosis-promoting factor (LPF), purified from materials extracted from agar in which *Bordetella pertussis* was grown, caused leukocytosis and an increased sensitivity to histamine when as little as 0.04 μg of protein was given intravenously to mice. LPF was adsorbed onto erythrocytes, causing hemagglutination. As little as 0.03 μg of LPF protein agglutinated chicken erythrocytes in 0.05 ml of a 0.5% suspension. The physicochemical, biological, and immunological properties indicated that leukocytosis, increased sensitivity to histamine in mice, and agglutination of erythrocytes are functions of a single substance.

The leukocytosis-promoting (LP) and histamine-sensitizing (HS) activities elicited by *Bordetella pertussis* are such unique biological properties that they are receiving the attention of not only of those who are working with the organism but also many other immunologists, allergologists, and pharmacologists (1, 4, 13, 14, 18, 20). Previously, we described the purification of the leukocytosis-promoting factor (LPF) from culture of *B. pertussis* by sequential processes of ammonium sulfate fractionation, zone electrophoresis, and sucrose density-gradient centrifugation, and demonstrated the homogeneity of the purified LPF by electrophoresis, ultracentrifugal analysis, immunoelectrophoresis, and electron microscopy (19). The purified LPF consisted of filamentous molecules (2 by 40 nm, mol wt 108,000) and caused leukocytosis in mice injected intravenously (i.v.) with 0.04 μg of LPF protein. In addition, the mice became highly sensitive to histamine. Purified LPF adheres easily to the membrane of blood and other cells of various origins, hence manifesting hemagglutination (HA) activity.

The present report describes the biological properties of LPF in more detail.

MATERIALS AND METHODS

Experimental animals. Female mice of the dd/N inbred strain, each weighing 18 to 20 g, were used.

LPF preparation. A preparation of LPF, purified by the procedures previously reported (19) and dissolved in 0.05 M phosphate buffer (pH 8.0) containing 0.5 M NaCl at a protein concentration of 0.2 mg/ml, was used throughout these experiments unless otherwise specified. The specific LP activity of the purified LPF (P-LPF) was approximately 24,000 U per mg of protein.

Determination of LP activity. LP activity was determined in the same way as described previously (19).

Determination of HS activity. Serial twofold dilutions of a specimen were inoculated i.v. into 5 to 10 mice in 0.2-ml doses. Three days later each mouse was challenged by i.v. injection with 1.0 mg of histamine base (histamine dihydrochloride, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Deaths were recorded at 4 h. The HS activity was expressed in HSD_{50}.

Determination of HA activity. Fresh chicken red blood cells (CRBC) from male white leghorn chickens were washed three times with 10 volumes of phosphate-buffered saline (PBS), pH 7.0. Twofold serial dilutions of each sample were made in PBS in 0.05-ml volumes in a row of wells in microtiter U plates (Cooke Engineering Co., Alexandria, Va.). An equal volume of a 0.5% CRBC suspension was added. The plate was agitated to mix CRBC thoroughly with the sample, and the mixture was incubated at room temperature for 2 h. One HA unit is defined as the least amount of the sample causing complete HA.

Determination of endotoxin activity. The method reported by Pieroni et al. (15) was followed. The mice, each pretreated by subcutaneous injection with 25 μg of actinomycin D (Schwarz/Mann, Orangeburg, N.Y.) contained in 0.2 ml, were injected intraperitoneally with different doses of P-LPF that had been boiled for 10 min. The largest dose was 500 μg of protein per mouse. The endotoxin activity was assessed from deaths occurring within 3 days. *B. pertussis* endotoxin, prepared by the phenol-water method of Westphal et al. (22), was used as a reference.

Antisera. (i) *Antibacterial serum:* Bacterial cells of *B. pertussis* phase I (strain Tohama) and phase III (strain Tohama) obtained from 2-day cultures on Bordet-Gengou medium were suspended in PBS containing 0.01% Merthiolate. The suspensions were stored for 1 year at 4 C. Each suspension was injected subcutaneously (s.c.) and later i.v. in 10 doses into male rabbits, each weighing about 3 kg. Each animal received a total of 4×10^{11} organisms. The rabbits were bled 2 weeks after the last injection. (ii) *Anti-LPF serum:* Two-milliliter amounts of partially purified LPF (SDGC-1, see Results) and P-LPF solutions, containing approximately 1 mg of protein per ml, were thoroughly emulsified in the same volume of oil adjuvant. Each emulsion was injected s.c. into the backs of rabbits. After 3 weeks, each rabbit was injected i.v. with 0.5 ml of each antigen solution without adjuvant. The animals were bled 2 weeks after the booster injection. (iii) *Anti-SRBC (sheep red blood cells) membrane-adsorbed LPF serum:* To 10 ml of an SRBC membrane suspension (0.5 mg of protein/ml) was added the same volume of a crude LPF preparation (NaCl extract; 1 mg of protein/ml). The mixture was incubated at 30 C for 90 min and centrifuged at $3,000 \times g$ for 10 min. The precipitate was suspended in 20 ml of PBS containing 0.5 M NaCl. After centrifugation and washing four times, the LPF-adsorbed membrane was suspended to its original volume. A 3-ml portion of this suspension was injected s.c. into the back of each rabbit. After 4 weeks, each rabbit was injected i.v. with 1 ml of the suspension. Two weeks after the second injection, the rabbits were bled.

Neutralization tests of LP and HS activities. A 2-ml amount of the twofold serial dilution of P-LPF solution was added to an equal volume of each undiluted antiserum. The solutions were mixed thoroughly and incubated at 36 C for 90 min. After incubation, each mixture was injected i.v. into 10 mice in 0.2-ml doses. Peripheral leukocytes were counted in 3 days. On the next day, each mouse was challenged by i.v. injection with 1 mg of histamine base to determine the HS activity from the fatality of each group.

Enzymes. Trypsin (×2 crystallized, Sigma Chemical Co., St. Louis, Mo.), α-chymotrypsin (×3 crystallized, Sigma), α-chymotrypsinogen (×6 crystallized, Sigma), Pronase (B grade, Calbiochem Inc., Los Angeles, Calif.), lysozyme (×3 crystallized, Sigma), phospholipase C (partially purified α-toxin of *Clostridium perfringens* donated by H. Sato of this Institute), neuraminidase (type V, Sigma), ribonuclease (×1 crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio), and deoxyribonuclease (×1 crystallized, Nutritional Biochemicals Corp.) were used.

Sucrose density-gradient centrifugation. The procedures for sucrose density-gradient centrifugation were as previously described (19).

CsCl density-gradient centrifugation. CsCl solutions with different densities were made in 0.05 M phosphate buffer (pH 8.0) containing 0.5 M NaCl. On top of 1.0 ml of a CsCl gradient (ρ = 1.500) in a cellulose nitrate tube, 0.9 ml of each CsCl solution, with densities of 1.350, 1.300, 1.250, and 1.200, were layered successively. Then, 0.4 ml of the P-LPF solution was added and the tubes were centrifuged at 38,000 rev/min for 24 h at 4 C in an SW65L Ti rotor with a Beckman L_4 ultracentrifuge. Fifteen-drop fractions were collected from the bottom of the tube, and the density of each fraction was determined. Then, each fraction with 0.5 ml of PBS added was used to determine absorption at 280 nm. Every two to four fractions were pooled and dialyzed overnight against 0.05 M phosphate buffer (pH 8.0) containing 0.5 M NaCl to determine the LP, HS, and HA activities.

Determination of protein content. Protein contents were determined with the copper-Folin reagent, with bovine serum albumin (0.2 mg/ml) as standard. The reaction mixture was made in 0.6-ml volumes, and readings were made in semimicro cells.

RESULTS

Sedimentation of LP, HS, and HA activity in the culture supernatant fluid by sucrose density-gradient centrifugation. Without undergoing any purification processes, the supernatant fluid of a culture of *B. pertussis* was centrifuged in sucrose density gradient to determine the sedimentation of the LP, HS, and HA activities. A 20-ml amount of supernatant fluid containing 0.46 mg of protein per ml was concentrated by dialyzing against Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) to a concentration of 3.2 mg of protein per ml. The concentration was centrifuged at $18,000 \times g$ for 15 min to remove insoluble materials, and the supernatant fluid was subjected to sucrose density-gradient centrifugation. The LP, HS, and HA activities of each fraction are shown in Fig. 1. All activities were contained in the same protein fraction sedimenting at a relatively high rate (fraction no. 14–16); the distribution patterns were similar in all the activities. The relative position of the peak of these activities was identical to that of P-LPF (5.6*S*).

Sedimentation of LP, HS, and HA activities in P-LPF by sucrose density-gradient centrifugation. Figure 2 shows the distribution of LP, HS, and HA activities in the fractions separated by sucrose density-gradient centrifugation of P-LPF. The sedimentation of the three activities coincided with that of the single protein peak. The specific activity per milligram of protein was on the same level throughout the peak (fraction no. 13–17): 25,600 (24,500–27,600) U of LP, 33,200 (29,000–35,400) HSD_{50} of HS, and 33,800 (29,000–35,200) U of HA activities. The amounts of P-LPF per unit of activity were approximately 0.04 μg of protein per LP unit, 0.03 μg of protein per HSD_{50}, and 0.03 μg of protein per HA unit.

LP, HS, and HA activities of samples obtained at different steps of purification.

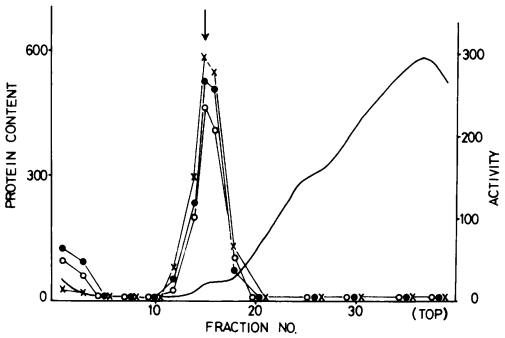

FIG. 1. *Sucrose density gradient centrifugation of the concentrated supernatant fluid of culture of B. pertussis. A 0.3-ml sample was layered on top of a 10 to 20% linear sucrose density gradient of 5.2 ml in 0.05 M phosphate buffer (pH 8.0) containing 1.0 M NaCl. After centrifugation at 60,000 rev/min for 17 h at 4 C in an SW65L Ti rotor with a Beckman L_4 ultracentrifuge, 0.13- to 0.14-ml fractions were collected. Symbols: ——, protein content (μg/ml); O, LP activity (U/ml); ●, HS activity (HSD_{50}/ml); X, HA activity (U/ml). The arrow indicates the sedimentation position of P-LPF (5.6S).*

Table 1 summarizes specific activities and recoveries (average of five runs) with respect to LP, HS, and HA activity of samples obtained at different steps of purification by the procedures described in the preceding report (19). The specific activity increased in parallel with specific HS and HA activity. The purification ratios obtained with respect to LP, HS, and HA activity were 470, 426, and 401, respectively; from those of the supernatant fluid, the recoveries were 20, 18, and 17%, respectively.

Sedimentation of LP, HS, and HA activities of P-LPF in CsCl density-gradient centrifugation. Sedimentation of LP, HS, and HA activities of P-LPF was investigated by CsCl density-gradient centrifugation. In terms of protein, P-LPF sedimented to the region with a density of approximately 1.230 (fraction no. 22), as did the LP, HS, and HA activities (Fig. 3).

Stability of LP, HS, and HA activity of P-LPF at different pH values. Portions (1 ml) of the P-LPF solution were dispensed into test tubes (0.8 by 6.0 cm) and adjusted to different pH values with a diluted NaOH or HCl solution. They were allowed to stand for 180 min at 25 C, and the activity of each was determined (Fig. 4). The stabilities of LP and HS activities were identical at different pH values; complete loss of activity occurred at pH below 2 and above 11. The HA activity behaved differently; loss of activity was seen below pH 6, where the LPF solution became turbid, indicating molecular aggregation. The turbid solution, if adjusted to pH 10 with NaOH, became clear immediately. The cleared solution was immediately adjusted to pH 7.5 with HCl, and HA activity was determined. Restoration of HA activity was observed when the initial pH was between 3 and 6. The restored activities were on the same level as LP and HS activities at the corresponding pH values. HA activity was irreversibly lost below pH2 and above 11, as were LP and HS activities.

Stabilities of LP, HS, and HA activities of P-LPF at different temperatures. The stabilities of LP, HS, and HA activities of P-LPF at different temperatures are shown in Fig. 5. P-LPF (pH 8.0) was dispensed in test tubes in 1.0-ml amounts. Each was heated for 15 min in water baths regulated at different temperatures, and the LP, HS, and HA activities were determined. No decreased activity resulted from exposure to temperatures below 56 C; all the activities decreased by about 50% at 70 C; complete loss of activity occurred at 80 and 100 C. The patterns of destruction were similar from one activity to another.

Sensitivity of LP, HS, and HA activity of P-LPF to $NaIO_4$. To each 0.5-ml portion of P-LPF, an equal volume of $NaIO_4$ solution of different concentrations was added. The mixtures were kept standing at 1 C for 18 h and then for an additional 30 min with 0.5 ml of a 10% glucose solution added to stop the oxidizing reaction of $NaIO_4$, after which LP, HS, and HA activities were determined. $NaIO_4$ at concentrations below 0.93 mM had no effect on any activity; decreased activity resulted from 2.8 mM or higher concentrations; complete loss of activity resulted from a concentration of 25 mM (Fig. 6A). To each 0.5-ml portion of P-LPF solution, 0.5 ml of a 50 mM $NaIO_4$ solution was

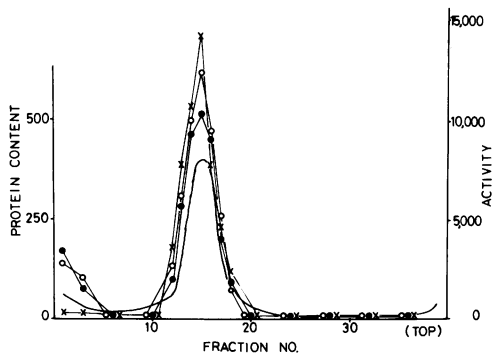

FIG. 2. *Sucrose density gradient centrifugation of P-LPF. See legend, Fig. 1, for centrifugal conditions. Symbols: ——, protein content (μg/ml); O, LP activity (U/ml); ●, HS activity (HSD_{50}/ml); X, HA activity (U/ml).*

TABLE 1. *LP, HS, and HA activities of the LPF samples obtained at each step of purification*

Fraction	Total protein (mg)	LP activity			HS activity			HA activity		
		Specific activity[a]	Ratio	Recovery of activity (%)	Specific activity[b]	Ratio	Recovery of activity (%)	Specific activity[c]	Ratio	Recovery of activity (%)
Supernatant fluid	960.0	50	1	100	85	1	100	86	1	100
NaCl extract	31.0	1,100	22	71	2,100	25	80	1,910	22	72
Zone electrophoresis	7.0	2,260	45	33	4,740	56	41	3,120	36	27
SDGC-1[d]	0.5	21,000	420	22	34,100	401	21	30,200	351	18
P-LPF	0.4	23,500	470	20	36,200	426	18	34,500	401	17

[a] Values shown indicate units of leukocytosis-promoting activity per milligram of protein.
[b] Values shown indicate mean dose of histamine-sensitizing activity per milligram of protein.
[c] Values shown indicate units of hemagglutination activity per milligram of protein.
[d] Partially purified LPF.

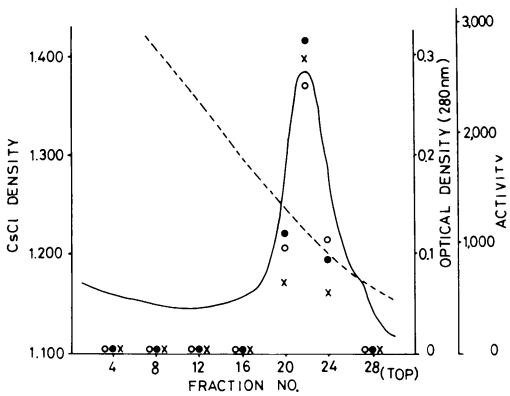

FIG. 3. *Sedimentation patterns of P-LPF in CsCl density gradient. Symbols: ——, optical density at 280 nm; -----, CsCl density (g/ml at 25 C); O, LP activity (U/ml); ●, HS activity (HSD_{50}/ml); X, HA activity (U/ml).*

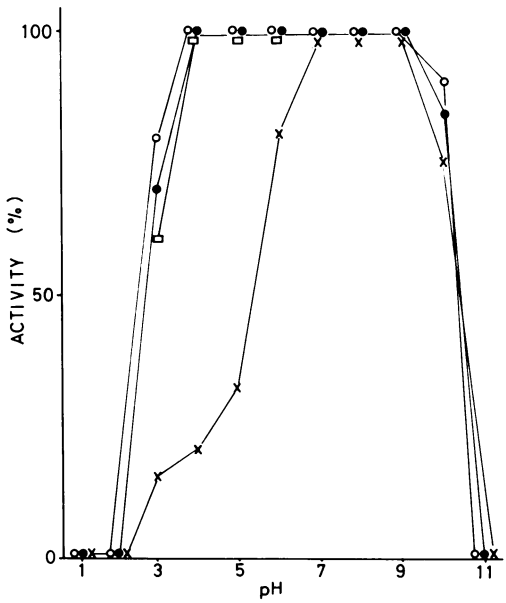

FIG. 4. *Stabilities of LP, HS, and HA activities of P-LPF at different pH values. Symbols: O, LP activity; ●, HS activity; X, HA activity; □, HA activity determined with the sample solubilized at pH 10 and then readjusted to neutrality. Each activity was determined after incubation at 25 C for 180 min.*

added. The mixtures were incubated at 37 C for different periods of time, and then 0.5 ml of a 10% glucose solution was added to each to stop the reaction and for determination of the three activities (Fig. 6B). All activities were completely lost by incubation for longer than 18 min. The rates of destruction of all the activities were on the same level at each incubation period.

Sensitivity of LP, HS, and HA activity of P-LPF to various enzymes. To each 0.5-ml portion of P-LF solution (200 μg of protein/ml) was added 0.5 ml of an enzyme solution (20 μg/ml in 0.05 M phosphate buffer, pH 7.2). The mixture of P-LPF with each enzyme had a pH of about 7.5, except for that with ribonuclease, which was dissolved in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.0. After incubation for 60 min at 37 C, the activity of each mixture was determined. The enzymes tested were trypsin, α-chymotrypsin, α-chymotrypsinogen, Pronase, lysozyme, phospholipase C, neuraminidase, ribonuclease, and deoxyribonuclease. Under the specified conditions, none of the enzymes tested reduced LP, HS, or HA activity of P-LPF.

Neutralization of LP and HS activity of P-LPF with different antisera. Neutralization tests of LP and HS activity of P-LPF were performed by using antisera with different properties (Table 2). With normal rabbit serum (no. 1) containing no neutralizing activity, injection with 0.05 μg of LPF resulted in a white blood cell (WBC) count of 21,000 and deaths of 20% of

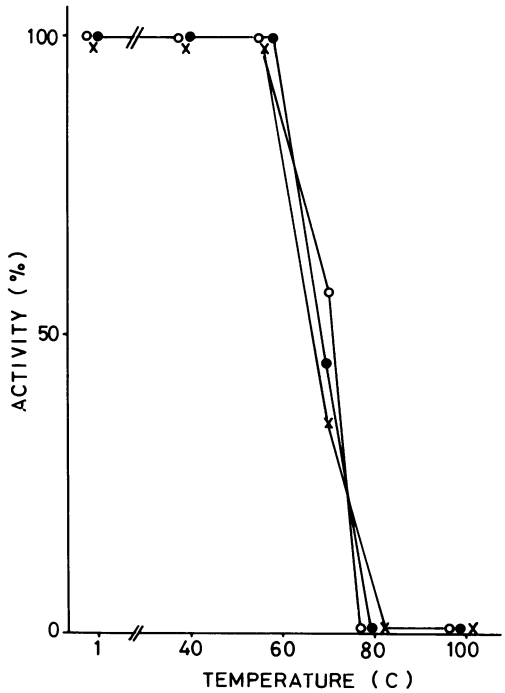

FIG. 5. *Stabilities of LP, HS, and HA activities of P-LPF at different temperatures. Symbols: O, LP activity; ●, HS activity; X, HA activity. Each activity was determined after incubation for 15 min at indicated temperatures.*

the mice challenged with histamine. With an increase in the dosage of LPF, the WBC population increased and the mortality from histamine administration became higher. Injection with no. 1 serum and 3.2 μg of LPF caused deaths in 40% of the mice before the WBC were counted. Similar results were obtained with antiphase III cell serum (no. 3), which contained no appreciable neutralizing activity. The other four sera, particularly anti-partially purified LPF serum (no. 4), anti-P-LPF serum (no. 5), and anti-SRBC membrane-adsorbed LPF serum (no. 6), markedly neutralized the LP and HS activities. High correlation was found between neutralization of LP activity and that of HS activity with any of these different serum preparations. When WBC population was at a normal level, no increased histamine sensitivity was found (italicized figures, Table 2). This may support the view that LP and HS activities are neutralized by the same antibody. Neutralization of HA activity was also attempted. However, it was found that all the sera, whether normal or immune, contained a nonspecific inhibitor of HA activity.

Adsorption of LPF molecules onto the cell membrane. The LPF molecules are adsorbed onto (and hence cause agglutination of) chicken, human, guinea pig, sheep, horse, cattle, duck, and mouse red blood cells, as well as white blood cells, e.g., spleen cells. They also agglutinate such cultured cells as HeLa-S_3, L, and Yoshida sarcoma cells. The LPF molecule (2 by 40 nm) appeared to be filamentous (19). The interaction between the filamentous molecules and the blood cell membrane was examined with an electron microscope (Fig. 7). The red cell membrane was prepared from fresh defibrinated sheep blood by the method of Dodge et al. (3). Portions (0.5 ml) of the red blood cell membrane suspension (0.1 mg of protein/ml) and of the P-LPF solution (0.1 mg of protein/ml) were mixed. The mixture was kept standing for 10 min at room temperature and stained negatively with phosphotungstic acid. The electron micrograph (Fig. 7) shows the filamentous LPF molecules being absorbed on the outer surface of the red blood cell membrane in a brushlike appearance.

Toxic activities of P-LPF. When injected intradermally into rabbits or guinea pigs, P-LPF did not elicit so-called thermolabile dermonecrotic toxicity, but, when injected intravenously into mice, it caused body weight decrease followed by death, mostly in 3 to 4 days. The mouse i.v. mean lethal dose of P-LPF, if determined in 5 days, was approximately 4 μg of protein. The lethal toxicity was not affected by heat treatment at 56 C for 30 min but was completely destroyed by exposure to 80 C for 15 min. P-LPF was examined for endotoxin activity by the method of Pieroni (15) in mice pretreated with actinomycin D. No endotoxin activity was demonstrated with a dose of P-LPF as large as 0.5 mg per mouse.

DISCUSSION

The physicochemically homogeneous LPF purified by us from a culture of *B. pertussis* contained not only the LP activity but also strong HS and HA activities (19). In the present investigation, studies were made on the relationships of these three activities. Without undergoing any purification processes, sedimentation of the LP, HS, and HA activities in culture supernatant fluid was scrutinized by sucrose density-gradient centrifugation. The three activities sedimented to the same relative position. In addition, the three activities resulting from such preparations, obtained at various steps of purification, were always recovered in the same fraction at the same level of recovery and the same rate of increase in specific activities on the protein basis. The behavior of these

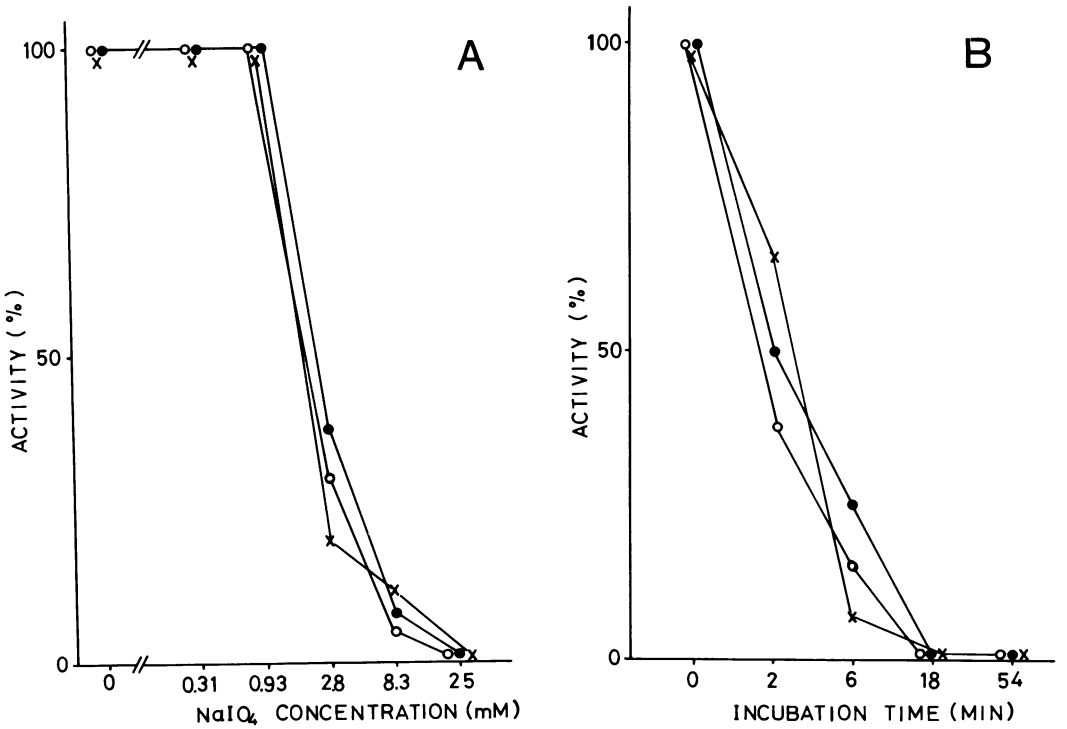

FIG. 6. *A, Sensitivities of LP, HS, and HA activities of P-LPF to different concentrations of $NaIO_4$. Symbols: O, LP activity; ●, HS activity; X, HA activity. Each activity was determined after incubation at 1 C for 18 h. B, Sensitivities of LP, HS, and HA activities of P-LPF to incubation with 25 mM $NaIO_4$ for different periods. Symbols: O, LP activity; ●, HS activity; X, HA activity.*

TABLE 2. *Neutralization of LP and HS activities of P-LPF with different antisera*

P-LPF (μg/mouse)	Antiserum[a]											
	No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
	WBC[b] (× 10^3)	Histamine[c] challenge (Fatality)	WBC	Histamine challenge	WBC	Histamine challenge	WBC	Histamine challenge	WBC	Histamine challenge	WBC	Histamine challenge
0.05	*21*	*20*	19	0	*27*	*10*	19	0	20	0	19	0
0.1	48	70	19	10	63	90	18	0	18	0	17	0
0.2	94	100	*25*	*20*	108	100	22	0	19	0	26	0
0.4	154	100	51	100	146	100	16	0	14	0	21	0
0.8	>200	100	123	100	>200	100	*23*	*20*	19	0	*20*	*0*
1.6	>200	100	151	100	>200	90	51	80	16	0	43	60
3.2	D[d]		>200	100	D		130	100	*29*	*10*	148	100

[a] Antiserum to: no. 1, control (normal rabbit serum); no. 2, phase I cells; no. 3, phase III cells; no. 4, partially purified LPF (SDGC-1); no. 5, P-LPF; no. 6, SRBC-membrane-absorbed LPF.

[b] Average white blood cell (WBC) count in 1 mm^3 of blood of 10 mice.

[c] Percent fatality from the histamine challenge.

[d] Dead before histamine challenge.

three P-LPF activities toward pH, temperature, oxidative reagents such as $NaIO_4$, and various preparations of antiserum, together with the electron micrograph, suggest that the three activities are elicited by the same LPF molecule. This is compatible with the suggestions of Morse and Morse (9) that LP and HS activities are elicited by the same molecule and that LPF

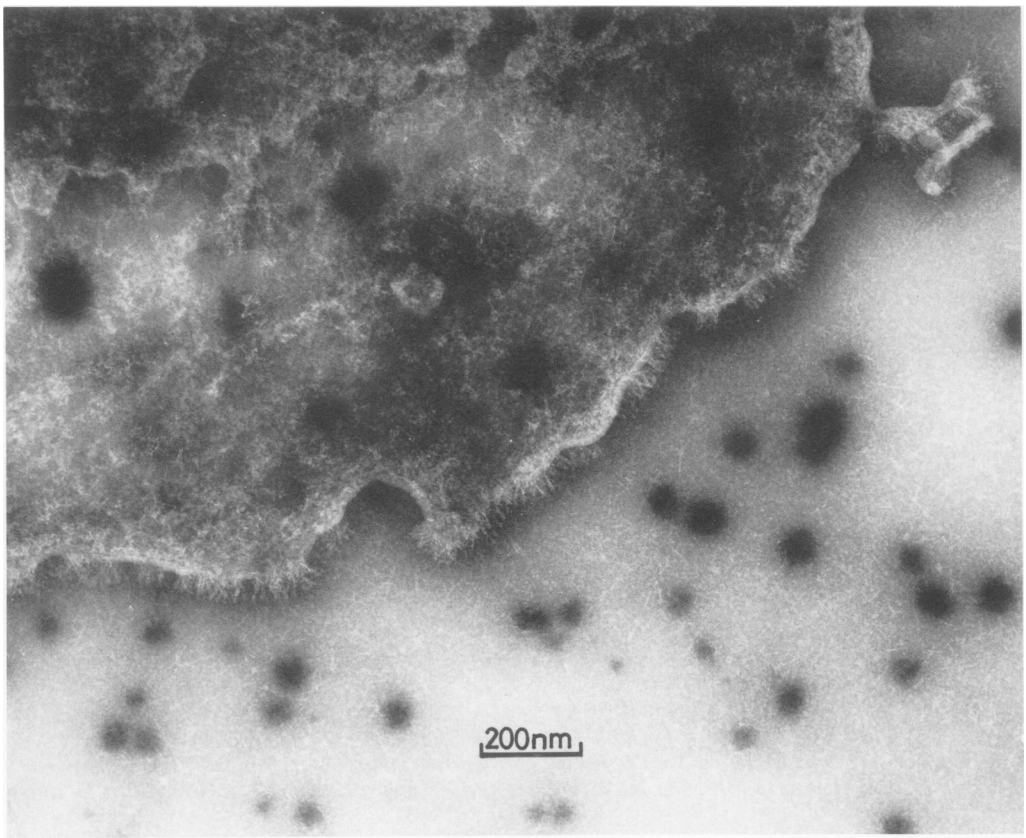

FIG. 7. *Electron micrograph of interaction of the LPF molecules with the sheep red blood cell membrane. A 1% solution of phosphotungstic acid in distilled water, adjusted to neutrality, was used for negative staining. Observations were made with a Hitachi HU-11B electron microscope.*

is adsorbed onto erythrocytes. The most highly purified HSF preparation of those obtained by Clausen et al. (2) contained LP activity. They also alluded to the same substance eliciting LP and HS activities. Some investigations were made on the HA of this organism, but none referred to the correlation with LP or HS activity (5, 7, 8, 21).

The present investigation revealed that the smallest unit of LP, HS, and HA activity released into the spent culture was the filamentous substance with a mol wt of about 110,000. The molecules tended to aggregate into polydisperse ones during purification processes, particularly in a solution with low ionic strength. Such unique biological activities of LPF have recently received the attention of research workers, particularly those in the field of immunobiology (14, 20; M. Seki and Y. Sato, Acta Pathol. Jap., in press). No endotoxin activity was found in P-LPF by the method of Pieroni. However, the sensitivity to endotoxin of the mice treated with actinomycin D in our experiments did not increase as much as he reported. This might depend upon the mouse strain.

The LPF molecules are adsorbed onto various cell membranes and hence elicit HA activity. An amount of P-LPF as small as 0.03 μg of protein caused HA with a 0.5% suspension of CRBC. Figure 7 shows that the adsorption of the LPF molecule onto the cell membrane is attained by both ends of the filamentous molecule. At the present time, the most likely explanation of the mechanism involved in leukocytosis and lymphocytosis caused by *B. pertussis* is that LPF produced by the organism is adsorbed onto the lymphocyte surface, causing disturbance in the normal migration flow in the lymphatic vessels, e.g., inhibition of lymphocytic emigration through the postcapillary venules into the lymphatic tissues (6, 10–12). Hence, retention of lymphocytes in the blood vessels may result. Such a disorder may be regarded as lymphocytosis. The actual adsorption of LPF

molecules onto the cell membrane observed by us may support the speculation described above.

Attempts at neutralizing LP, HS, and HA activities by various immune sera revealed that rabbit serum, containing nonspecific hemagglutinin inhibitors at high concentration, inhibits nonspecifically the HA activity of LPF. The LP-, HS-, and HA-neutralizing activities of the sera (after removing the nonspecific inhibitor) are now under investigation.

The correlation between the red cell membrane-adsorbed protective antigen reported by Pillemer (16, 17) and the LPF preparation causing the LP, HS, and HA activities studied by us will logically constitute the next problem to be solved.

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