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Leucocytosis-Promoting Factor of *Bordetella pertussis*

I. Purification and Characterization

Y. SATO AND H. ARAI

First Department of Bacteriology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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The leucocytosis-promoting factor was purified from the supernatant fluid of spent cultures of *Bordetella pertussis* on solid medium. After precipitation at 67% saturation of ammonium sulfate, the leucocytosis-promoting factor was extracted with a 1.0 M NaCl solution. Purification was accomplished by starch block electrophoresis and sucrose density gradient centrifugation. The purified preparation contained a high leucocytosis-promoting activity, and as small an amount as 0.04 μg of protein induced leucocytosis in mice. About 520-fold purification was attained, with a recovery of about 25% on an activity basis. The leucocytosis-promoting factor was composed solely of filamentous molecules of about 2 by 40 nm in size, with a sedimentation coefficient of approximately 5.5*S* and a molecular weight of 108,000. It was insoluble in water but partially soluble in 1.0 M NaCl solution, and consisted mainly of protein, with some carbohydrate, lipid, and phosphorus.

Bordetella pertussis cells or cell extract, when injected intravenously into animals, cause marked increase in the population of small lymphocytes in the circulating blood (4, 11). Little is known about the agent causing the leucocytosis. Attempts have been made to isolate the agent from the culture supernatant fluid or cell extract (7, 13, 17, 18), but no one has succeeded in obtaining it in a pure state. Morse and Bray (13) detected most of the leucocytosis-promoting factor(s) (LPF) in the culture supernatant fluid harvested after the maximal growth. They found also that the leucocytosis was caused only by the fraction containing filamentous substance separated by CsCl density gradient centrifugation (10).

This report deals with purification of the LPF by zone electrophoresis and by centrifugation in sucrose density gradient from the supernatant fluid of spent cultures on a solid medium after the grown organisms have been removed. Some physicochemical properties of purified LPF will also be described.

MATERIALS AND METHODS

Organisms. *B. pertussis* strain Tohama phase I, being used for production of pertussis vaccine by most manufacturers in this country, was used. The strain produced a larger amount of LPF than strain 3779B or any other strain tested. Seed cultures were grown at 36 C for 20 hr on Bordet-Gengou agar medium containing 20% defibrinated bovine blood.

LPF production. Modified Cohen-Wheeler agar slants were inoculated from seed cultures and then incubated at 36 C for 72 hr.

The modified Cohen-Wheeler medium consisted of the following ingredients per 10 liters: agar powder (Eiken), 185.0 g; Casamino Acids (Difco, technical), 100.0 g; yeast extract (Difco), 20.0 g; activated charcoal (Takeda Pharmaceutical Co.), 20.0 g; soluble starch (Difco), 15.0 g; L-cystein-hydrochloride (Merck), 0.3 g; NaCl, 25.0 g; KH_2PO_4, 5.0 g; $MgCl_2 \cdot 6H_2O$, 4.0 g; $CaCl_2$, 100 mg; $FeSO_4 \cdot 7H_2O$, 100 mg; and $CuSO_4 \cdot 5H_2O$, 70 mg. The mixture was adjusted to *p*H 7.2, dispensed into Roux bottles in 50-ml portions, and autoclaved at 121 C for 40 min.

Starting material for purification of LPF. The bacterial growth was scraped off the slants, and then the remaining medium containing LPF was frozen for 24 hr, thawed in a water bath at 30 C, and centrifuged at 20,000 × *g* for 20 min at 4 C to remove the supernatant fluid. About 4.5 liters were obtained from 10 liters of medium.

Assay for LPF. Each of serial twofold dilutions of a sample was inoculated intravenously into five mice at a dose of 0.2 ml. Female mice of the dd/N strain, weighing 18 to 20 g, were used. Three days after the injection, total white blood cells (WBC) per cubic millimeter were counted with a hemocytometer. A plot of the log WBC count versus log LPF dose gave a linear dose response curve within a range of the WBC count from 3.2×10^4 to 2.4×10^5 (Fig. 1). The slope of the curve did not differ significantly from one preparation of LPF to another. The test for heterogeneity variance demonstrated that the magnitudes of variance of log numbers of WBC at different doses were on the same level. One unit of

LPF activity was arbitrarily defined as the amount giving rise to a total WBC population of 32,000/mm³ (Fig. 1).

Starch block electrophoresis. A 10-ml portion of a sample (3 mg of protein/ml) was applied at the center of a starch block (50 by 12 by 1.5 cm) saturated with 0.2 M phosphate buffer, *p*H 8.0. Electrophoresis was carried out at 65 to 70 ma for 44 hr at 4 C. The block was cut into 50 1-cm sections; each section was extracted with 10 ml of 0.05 M phosphate buffer, *p*H 8.0, containing 1.0 M NaCl.

Sucrose density gradient centrifugation. A 0.2-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 (*p*H 8.0) containing 1.0 M NaCl. After centrifugation at 60,000 rev/min for 17 hr at 4 C in an SW-65L-Ti rotor with a Beckman L_4 ultracentrifuge, 12-drop fractions or 0.13- to 0.14-ml fractions were collected manually.

Electrophoresis on cellulose acetate membrane. A 10-μliter portion of purified LPF (0.8 mg of protein/ml) was applied at the center position on a piece of cellulose acetate membrane (2.0 by 10 cm). The buffer used was 0.1 M phosphate at *p*H 6.0, 7.0, or 8.0. Electrophoreses were run at 1.4 ma for 60 min at 4 C. Amido black 10B was used to locate the protein bands.

Immunoelectrophoresis. Electrophoresis of purified LPF was performed in 1% agarose (Nakarai Chemicals Co. Ltd.) gel made in 0.05 M phosphate buffer, *p*H 8.0, containing 0.5 M NaCl. A current of 4.5 ma per cm was applied for 90 min at 4 C, the antiserum was added to each trough, and the slides were incubated at 4 C for 3 days.

Ultracentrifugal analysis. A 0.3% solution, based on protein determination, of purified LPF made in 0.05 M phosphate buffer (*p*H 8.0) containing 0.2 M NaCl was spun at 63,650 rev/min at 30 C in a Beckman model E analytical ultracentrifuge. Photographs were taken every 8 min after reaching the maximal speed.

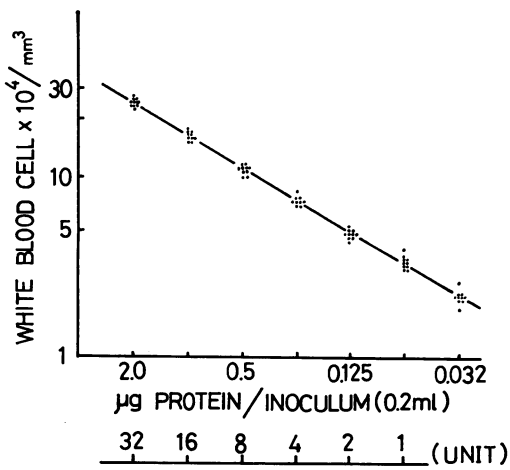

FIG. 1. *Relationship between the dose of LPF and the number of white blood cells. Each of seven serial twofold dilutions of a purified LPF preparation was injected intravenously into 10 mice at a 0.2-ml dose. A linear correlation between the log dose and the log number of WBC was obtained.*

Molecular weight estimation. Martin and Ames (9) determined sedimentation coefficients and approximate molecular weights by sucrose density gradient centrifugation. Portions (0.1 ml) of purified LPF were centrifuged in 10 to 20% sucrose density gradient for different periods of time to estimate the sedimentation coefficient and the molecular weight. Bovine serum albumin, with a sedimentation coefficient of 4.0*S* and a molecular weight of 67,300, dissolved in phosphate-buffered saline (*p*H 7.2) at a concentration of 0.2 mg/ml was used as a reference.

Electron microscopy. A drop of a solution of purified LPF (0.2 mg of protein/ml) was applied on each grid that had been covered with parlodion film stabilized with carbon; it was then allowed to dry partially for a few minutes at room temperature. A 1% solution of phosphotungstic acid in distilled water, adjusted to neutrality, was used for negative staining. Observations were made with an Hitachi HU-11B electron microscope.

Chemical analyses. Total nitrogen was estimated by the indophenol method (1); protein content by the method of Lowry et al. (8) with bovine serum albumin as the standard; total sugar content without prior hydrolysis by the use of anthrone (19); lipid content by the method of Folch (6); phosphorus contents of samples digested with perchloric acid by the method of Allen (2); aminosugar content by the method of Dische (5); ribonucleic acid (RNA) content by the method of Webb (20); and deoxyribonucleic acid (DNA) content by the diphenylamine reaction (3).

RESULTS

Preparation of starting material for fractionation of LPF. To 8,000 ml of the supernatant fluid obtained by processing the agar medium in which *B. pertussis* had grown, 4,000 g of solid ammonium sulfate was added. The mixture was adjusted to neutrality with ammonium hydroxide. The resulting precipitate was allowed to settle for 15 hr, and then the clear supernatant fluid was decanted. The precipitate was centrifuged at 10,000 × *g* for 15 min and suspended in 80 ml of 0.05 M phosphate buffer, *p*H 8.0, containing 1.0 M NaCl. The suspension was homogenized thoroughly with a Teflon homogenizer and centrifuged at 14,000 × *g* for 20 min. The residue was extracted five more times with 30 ml of buffer each time, and the extracts were combined (extract). About 70% of the LPF activity found in the starting material was recovered, and the specific activity increased about 20 times (Table 1). Storing the extract at −20 C for several weeks caused no marked inactivation of the LPF activity.

Zone electrophoresis of the extract. A 10-ml amount of the extract obtained from the agar medium was dialyzed against 0.2 M phosphate

TABLE 1. *Recovery and specific activity at various stages of purification of LPF*

Fraction	Protein		Specific activity		Activity	
	Total (mg)	%	Units/mg of protein	Ratio	Total units	%
Supernatant fluid	3,840	100	47	1	180,480	100
Extract	130	3.4	846	18	127,400	71
Z-F	26.5	0.7	2,350	50	62,275	35
SDGC-I	2.4	0.06	23,000	490	54,144	30
Purified LPF	2.0	0.05	24,440	520	48,880	27

buffer (*p*H 8.0) for 20 hr and then was subjected to electrophoresis in a starch block. Three main protein peaks were separated. Most LPF activity was found in the cathodic fraction; some remained at the starting position (Fig. 2). Fractions 15 to 17 were pooled and concentrated by dialysis against Ficoll to 1.5 mg of protein/ml. This fraction, labeled Z-F, contained 35% of the initial LPF activity and had 2,350 U per mg of protein and a specific activity about 50 times higher than the starting material (Table 1).

Sucrose density gradient centrifugation of Z-F A 0.2-ml amount of Z-F (1.5 mg of protein/ml) was resolved into three protein peaks by sucrose density gradient centrifugation (Fig. 3). The LPF activity was found only in the first sedimenting peak. Fractions 13 to 17 were collected and concentrated with Ficoll to about 0.5 mg of protein/ml (SDGC-I). About 90% of inactive protein in Z-F was removed. LPF activity of SDGC-I was 490 times as high as that of the supernatant fluid (Table 1). To diminish the sucrose content, 1 ml of SDGC-I was dialyzed for 3 hr against 0.05 M phosphate buffer (*p*H 8.0) containing 1.0 M NaCl. After dialysis, a 0.2-ml portion (0.5 mg of protein/ml) was centrifuged again in sucrose density gradient under the same conditions. LPF activity was associated only with the main protein peak (Fig. 4). Fractions 13 to 17 were collected (purified LPF).

Table 1 summarizes the recovery figures and the specific activities obtained at various stages of purification of LPF. Purified LPF possessed a specific activity about 520 times higher than that of supernatant fluid; the recovery in activity was 27%. As small an amount as 0.04 μg of protein contained 1 LPF unit.

Electrophoreses of purified LPF on cellulose acetate membrane. Purified LPF behaved as a homogeneous protein in cellulose acetate membrane electrophoresis at *p*H 7.0 or 8.0 (Fig. 5). At *p*H 6.0, however, a small amount of nonmigrating protein was observed. When the LPF preparation was adjusted to *p*H 6.0 or below, fine precipitate appeared, possibly from molecular aggregation.

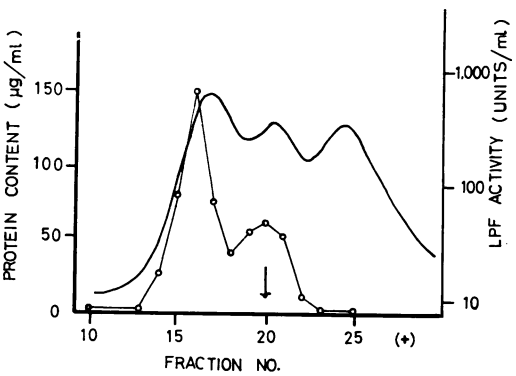

FIG. 2. *Starch block electrophoresis of extract. The arrow represents the original point. Symbols:* (——) *protein content;* (O——O) *LPF activity.*

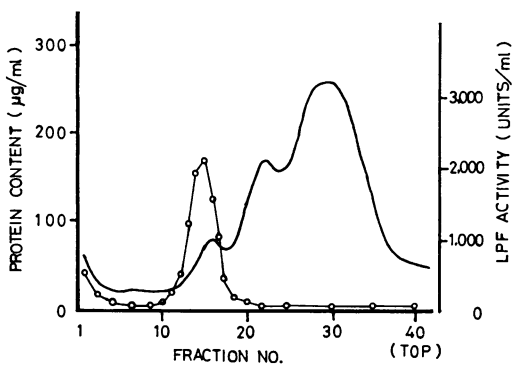

FIG. 3. *Sucrose density gradient centrifugation of Z-F. Symbols:* (——) *protein content;* (O——O) *LPF activity.*

Immunoelectrophoreses of LPF preparations at different purification steps. Immunoelectrophoreses of extract, SDGC-I, and purified LPF were performed (Fig. 6). There were at least three precipitation lines with extract and SDGC-I against anti-whole bacterial cell rabbit serum or anti-Z-F rabbit serum; a single precipitation line was observed with purified LPF against anti-SDGC-I rabbit serum or anti-whole bacterial cell horse serum of a high agglutinin titer.

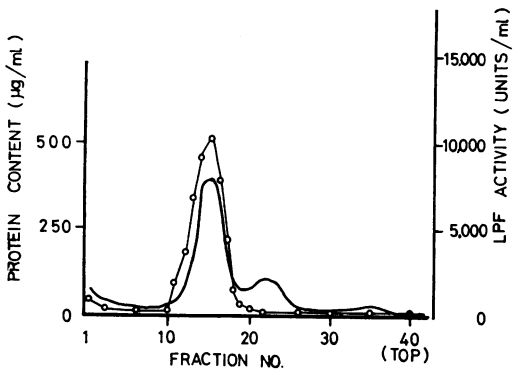

FIG. 4. *Sucrose density gradient centrifugation of SDGC-I. Symbols: (——) protein content; (O——O) LPF activity.*

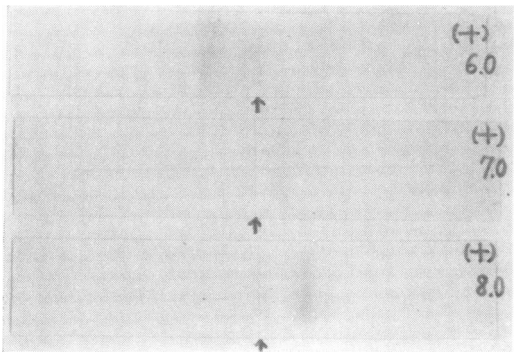

FIG. 5. *Electrophoresis on cellulose acetate membranes at pH 6.0, 7.0 and 8.0 of purified LPF. The arrows indicate the points of application of samples.*

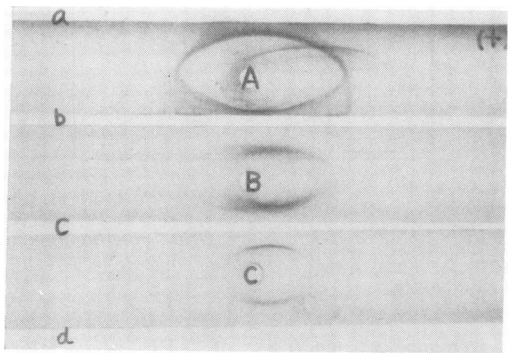

FIG. 6. *Immunoelectrophoresis of different preparations. Antigen: A, extract (1.2 mg of protein/ml); B, SDGC-I (0.6 mg of protein/ml); C, purified LPF (0.6 mg of protein/ml). Antiserum to: a, whole bacterial cell (25,600); b, Z-F (800); c, SDGC-I (<100); d, whole bacterial cell (800,000). Figures in parentheses after the antisera show agglutinin titers.*

Ultracentrifugal analysis. A single boundary with an $S_{20,w}$ of 5.6 was observed with purified LPF in ultracentrifugation. Figure 7 shows the picture taken in 40 min.

Molecular weight estimation. LPF and bovine serum albumin were centrifuged separately in sucrose density gradient for different periods of time, and the respective linear curves were obtained as shown in Fig. 8. The sedimentation coefficient of purified LPF was calculated by the equation of Martin and Ames (9), $S_1/F_1 = S_2/F_2$, where S_1 and S_2 are sedimentation coefficients

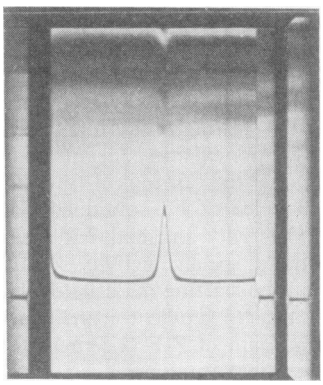

FIG. 7. *Analytical ultracentrifugation pattern of purified LPF.*

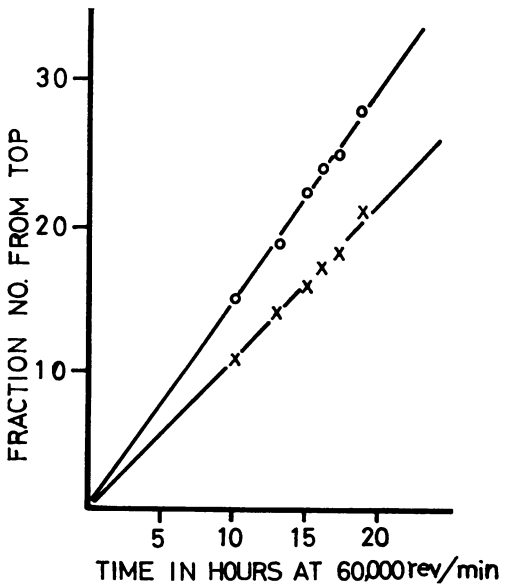

FIG. 8. *Sedimentation of purified LPF and bovine serum albumin in 10 to 20% sucrose density gradient at 4 C. Each point represents the fraction number giving the highest protein content. Symbols: ×, bovine serum albumin; O, purified LPF.*

and F_1 and F_2 fraction numbers of the protein peaks of purified LPF and bovine serum albumin, respectively. The sedimentation coefficient of LPF calculated from six determinations was 5.54*S*, with a standard deviation of ±0.08. This is in good agreement with the value obtained by analytical ultracentrifugation (Fig. 7). By the formula, S_1/S_2 = (molecular weight$_1$/molecular weight$_2$)$^{2/3}$, the molecular weight of LPF was estimated to be approximately 108,000.

Chemical analyses. Purified LPF was dialyzed against distilled water for 4 days and lyophilized. The lyophilized LPF was hardly soluble in water, but partially soluble in the presence of 1.0 M NaCl. The chemical composition of a lyophilized preparation of LPF was as follows: total nitrogen, 7.5%; protein, 46.8%; sugar, 24.5%; aminosugar, 4.5%; lipid, 17.5%; phosphorus, 1.8%; DNA, 0%, and RNA, 0%.

Electron microscopy. Purified LPF was stained negatively and examined with an electron microscope (Fig. 9). It was revealed that purified LPF consisted solely of uniform filamentous molecules of about 2 by 40 nm.

DISCUSSION

B. pertussis phase I cells provoke a variety of physiological changes in experimental animals (16), including leucocytosis. Recently, Morse et al. (11, 12, 14, 15) studied the mechanisms involved in development of leucocytosis in mice injected with pertussis cells. Attempts have been made to isolate the leucocytosis-promoting agent(s), but no satisfactory purification has ever been accomplished.

We attempted to isolate LPF from *B. pertussis* and characterize it. A larger amount of LPF was produced in a solid medium than in a liquid medium; sonic treatment of the bacterial cells released no appreciable amount of LPF. The supernatant fluid of the spent agar slant culture was highly potent in inducing leucocytosis; the activity on protein basis was two or three times higher than that of the intact bacterial cells or supernatant of liquid culture.

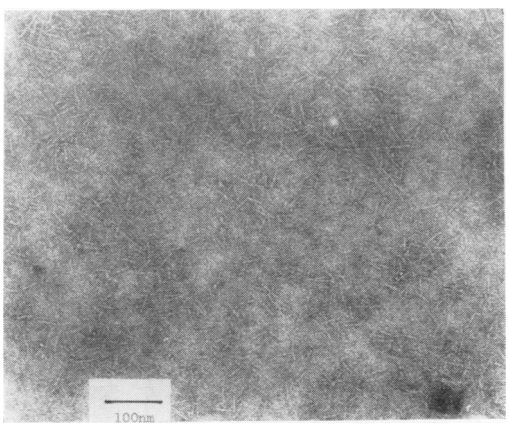

FIG. 9. *Electron micrograph of purified LPF.*

By starch block electrophoresis and centrifugation in sucrose density gradient in the presence of a high content of sodium chloride, physicochemically homogeneous LPF was obtained. Purified LPF consisted mainly of protein, with some carbohydrate, lipid, and phosphorus. By sucrose density gradient centrifugation, a sedimentation coefficient of 5.54*S* and a molecular weight of about 108,000 were calculated.

Morse and Bray (13) stated that LPF tended to be adsorbed readily onto particulate materials below neutrality; low salt concentration also favored aggregation of LPF, resulting in formation of hardly soluble precipitate. LPF purified by us was also readily agglutinable and precipitable at low salt concentrations or at low *p*H values. Cellulose acetate membrane electrophoresis at *p*H 6.0 indicated possible partial aggregation of LPF, being immobile at the starting point.

The molecules of LPF were filamentous, with a diameter of approximately 2 nm and a length of 40 nm (Fig. 9). This is in accord with the finding by Morse and Morse (10). The same authors alluded to a possibility that LPF and the histamine-sensitizing factor are one and the same entity. Iwasa et al. (7) also reported a high histamine-sensitizing activity possessed by their LPF preparation. Okuyama et al. (17), on the other hand, reported that LPF is an entity distinct from the histamine-sensitizing factor. Our LPF, having behaved as a homogeneous substance in electrophoresis, sedimentation, immunoelectrophoresis and electron micrography, possessed a high histamine-sensitizing activity in accord with Morse and Morse (10), and in addition to that a high hemagglutinating activity. The biological properties possessed by our LPF preparation will be detailed elsewhere.

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