**LYMPHOCYTOSIS-PROMOTING FACTOR PRODUCED BY
BORDETELLA PERTUSSIS
PARTIAL PURIFICATION AND CHARACTERIZATION***

Absolute lymphocytosis often occurs in children suffering from whooping cough and also in men and experimental animals injected with certain preparations of pertussis vaccine. Attempts have been made to separate the entity responsible for the lymphocytosis from pertussis cells, but none succeeded in obtaining the material practically free of other known biological activities.

Kurokawa, Iwasa and Ishida (1965) reported a toxic factor produced by pertussis bacilli showing such late-appearing toxicities (LAT) as late body-weight loss and late death of mice. During the course of purification of the toxic factor, it was noted that every fraction showing LAT for mice is accompanied by lymphocytosis-promoting activity (LPA). Since there were no evidences against the assumption that one and the same entity is responsible for both LAT and LPA, the authors designated the factor as lymphocytosis-promoting factor (abbreviated to LPF) (Iwasa et al., 1966) and initiated purification and characterization of this factor.

This report deals with the methods of purification and some physicochemical properties of LPF preparations, some of which have been used in our previous experiments (Ishida, 1968, Kurokawa et al., 1968).

B. pertussis strain, No. 3779B (Kurokawa, Iwasa and Ishida, 1965) was cultured in a modified Cohen-Wheeler's medium containing 0.04 % activated charcoal and 0.0001 % DL-tryptophan at 37 C for 15 to 20 hr on a shaker. The culture was added with 0.01 % merthiolate and kept for a few days at room temperature to be sterilized. All the subsequent manipulations were carried out at 0-4 C unless otherwise specified. The cells were removed by centrifugation at 10,000 rpm for 15 min. The supernatant was saturated with ammonium sulfate and allowed to stand overnight. The resulting precipitate was suspended in saline (0.15 M NaCl) of one-hundredth the original volume, which was dialyzed against saline. The insoluble material, designated as the Pr I fraction, was centrifuged at 10,000 rpm for 20 min, washed once and resuspended in saline of the same volume as that before dialysis, and tested for the biological activities.

The Pr I fraction suspended in saline was extracted three times at room temperature for 10 min with 0.02 M tris-buffer (pH 9.0) containing 0.2 M NaCl with stirring; the first and the second extractions were made with 5 volumes and the third with 2.5 volumes of the buffered saline. Each extract was taken by centrifugation at 10,000 rpm for 20 min.

To the combined extract was added ammonium sulfate to attain 70 % saturation, and the resulting precipitate was dialyzed against saline. The insoluble residue (Pr II fraction) was extracted again three times with 0.02 M tris-buffer (pH 9.0) containing 0.4 M NaCl under the above-described conditions except that the volumes of the ex-

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tractant were one-fourth for the 1st and 2nd extractions and one-fifth for the 3rd extraction.

The combined extract was spun in an ultracentrifuge at 100,000 g for 60 min and the supernatant was concentrated by suction through cellophane membrane to a protein concentration of about 0.03 %. Forty ml of the concentrate was subjected to gel filtration through a column (2.7×60 cm) of Sephadex G-100 equilibrated with 0.02 M tris-buffer (pH 9.0) containing 0.4 M NaCl at a flow rate of 15 ml/hr.

The LAT* was eluted in the fractions of the retarded large protein peak (Fig. 1). The recovery of LAT in the peak fractions (tube number, 18–20) was about 50 % of the material loaded; the total recovery was nearly 100 %. The peak fractions were pooled and it was rechromatographed under the same conditions. A single protein peak was obtained and nearly 100 % of the activity in the material loaded was recovered in the peak.

D₀D₄** and a minimum dose producing lymphocytosis in mice of the peak fractions when injected intravenously were estimated to be 0.05 to 0.1 µg in protein (Fig. 2). The recovery of protein in the peak fractions was 0.2 % of that in the original culture supernatant, while that of sugar 0.001 % (Table 1) in glucose equivalent. The sugar content was only one-sixth of the protein content. By isoelectric precipitation of LPF, a large amount of sugar was eliminated without loss of both LAT and LPA.

Both the activities decreased apparently by the treatment of LPF with such proteases as trypsin and pronase at 31°C for 60 min and was completely destroyed by the treatment with 0.2 % formalin within 7 days resulting in formation of a toxoid with a high antigenicity. Although further investigation may be needed, the above results may indicate that at least the main component responsible for the activities is of protein nature.

The LPF obtained by gel filtration formed a single precipitation line at a concentration of 0.01 % (in protein) in an agar against anti-LPF mouse serum*** or an anti-LPF rabbit serum (APr1-1) (Ishida, 1968), while no line was seen against anti-whole cell rabbit serum (agglutinin titer, 1200 units).

Both LAT and LPA were completely neutralized with the anti-LPF rabbit serum, but not with the anti-whole cell rabbit serum.

A sedimentation coefficient of a preparation of LPF (the concentrations was 0.05 for protein and 0.004 % for carbohydrate) obtained by the gel filtration was about 5S.

Fig. 3 shows the changes in the number of peripheral leukocytes and in the body weight of the mice inoculated intraperitoneally with about 4 LD₅₀ (10 µg protein) of the material obtained by the first gel filtration. A striking loss of body weight and a remarkable increase in number of leukocytes, especially of small lymphocytes, were observed. All the mice died in 4 to 6 days after inoculation. The lymphocytosis invariably starts within 1 day and reached its plateau in 3 to 4 days after the inoculation, while the body weight decrease usually began on the 3rd day.

The leukocytosis at an early stage consisted largely of an increased number of mature small lymphocytes. Polymorphonuclear leukocytes (PMN) also increased, but the increase of PMN took place later than that of lymphocytes. The magnitude of

* LPF was assayed routinely by measuring LAT.

** D₀D₄ is an absolute activity unit of LPF proposed by Ishida (1968), who has taken LAT as the response, and difference zero dose estimated from BWD₄₋₂ (see the footnote in Fig. 1).

*** Prepared by immunizing the animals with a formal toxoid made from a gel-filtered material

Fig. 1. Gel filtration through Sephadex G-100 column

- Protein content in bovine serum albumin equivalent as determined with the Folin-Ciocalteu reagent (Oyama and Eagle, 1956).
- Sugar content in glucose equivalent as determined by primary cysteine-sulfuric reaction (Dische, Z, 1955).
- ×—× Activity in terms of LAT.

* a measure of the body-weight response to LPF proposed by Ishida (Ishida, 1968); (body weight on the 4th day)-(body weight on the 2nd day).

Fig. 2. Regressions for LAT and LPA on log dose of an LPF preparation.

Groups of 10 female mice (conventional ddS strain, 17-20 g) were inoculated intravenously with various doses in 0.2 ml and weighed every day for calculation of BWD_{4-2} (see the footnote for Fig. 1). Other groups of five mice were inoculated and, one and four days later, blood from each mouse was taken from the tail vein for counting the peripheral leukocyte,

- No. of peripheral leukocytes one day after inoculation.
- No. of peripheral leukocytes four days after inoculation.
- BWD_{4-2}.

Table 1. Protein and sugar contents, and activity of fractions at each step of the purification

	Protein		Sugar		Activity	
	Total	%	Total	%	%	Specific activity (on protein basis)
Culture fluid (42 l)	2,436	100	67,200	100	100	1
Pr I fraction	715	29			100	3.4
Pr II fraction	234	9.6			90	9.4
Supernatant after ultra-centrifugation at 100,000 g	60.7	2.5	3.5	0.05	50	20
Peak fractions obtained at primary gel-filtration step	10.7	0.4	1.1	0.002	30	75
Peak fractions obtained at secondary gel-filtration step	4.9	0.2	0.8	0.001	20	100

Fig. 3. Changes in body weight, spleen weight and the number of the peripheral leukocytes of mice after intraperitoneal inoculation with a preparation of LPF.

Twenty-five mice (conventional ddS strain, 15-17 g) were weighed every day and differences from the starting body weight were taken. Four mice were killed every day for counting the peripheral leukocytes and weighing the spleen.

- Left figure Changes in the weights of the body and the spleen.
- Body weight of control mice.
 - Body weight of inoculated mice.
 - △---△ Spleen weight of control mice.
 - ▲—▲ Spleen weight of inoculated mice.
- Right figure Changes in the number of the peripheral leukocytes.
- Total leukocytes of control mice.
 - Total leukocytes of inoculated mice.
 - ⊙—⊙ Small lymphocytes of inoculated mice.
 - ⊖—⊖ Polymorphonuclear leukocytes of inoculated mice.
 - ⊕—⊕ Sum of large lymphocytes and monocytes of inoculated mice.

increase of PMN varied from one experiment to another.

Fig. 3 also shows that the weight of the spleen of the mouse clearly decreased after the inoculation, contrary to the observation of Morse (1965) who used a whole cell vaccine. The microscopic observations of the spleen done by us were in good accordance with his observation; the majority of lymphocytes in the follicles disappeared, there was a moderate increase of PMN in red pulp, and mitotic figures were no more numerous than in the controls.

No data against the identity of the chemical entities responsible for LPA and LAT have been obtained with preparations of LPF even after much purification.

In view of the well-known multiplicity of toxic actions of pertussis bacilli, the relationships of LPF to other known toxic substances, particularly the endotoxin, the heat-labile toxin and the histamine-sensitizing factor (HSF), was to be carefully examined.

Besides differences in biological properties established by Ishida (1968), a) the LPF fractions obtained from the gel filtration contained little heptose, while the endotoxin contains a relatively large amount of heptose (McClennan, 1960, Yoshikawa and Fukumura, 1966) and b) the activity of LPF was reduced apparently with proteases.

Previous results from our laboratory suggested that LPF and the heat-labile toxin were different entities (Ishida, 1968). A gel-filtrated preparation of LPF obtained in the present investigation produced no demonstrable dermatonecrosis in guinea-pigs, rabbits and suckling mice with doses of 11, 10 and 2 μ g, respectively, of a gel-filtered material. According to Sato et al.*, the guinea-pig inoculated intravenously with a purified preparation of the heat-labile toxin developed congestion and hemorrhage in the systemic lymph nodes and also in other organs. On the macro- or microscopic examination in mice, our LPF material produced neither congestion nor hemorrhage in lymph nodes or other tissues even with a lethal dose.

Every LPF fraction from different steps of purification showed rather strong histamine-sensitizing activity (HSA). Although some data showed inconsistent ratios of LAT to HSA in different fractions, it is difficult to evaluate the data precisely because of the insufficient accuracy of the quantitative determination of HSA. Kura-tsuka et al. (1967) noted that purified preparations of HSF contained relatively low LAT or LPA. Further purification of LPF is necessary to correlate LPF with HSF.

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