Attempts at Isolation of Lymphocytosis-Producing Factor from Supernatant Fluids of *Bordetella pertussis* Cultures[1] (34552)

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An increased blood lymphocyte count is seen during the course of infection due to *Bordetella pertussis* (1) as well as in response to pertussis vaccine in human beings (2). Extracorporeal irradiation of the blood or lymph (ECIB or ECIL) produces a marked lymphocytopenia (3). ECIB produces a depletion of lymphocytes in the lymph nodes, thymus, and spleen (4). Irrespective of the length of ECIB, about 50% of cortical small lymphocytes remain in the lymph nodes (5). Our interest in *B. pertussis*-induced lymphocytosis stemmed from the possibility that this agent might mobilize these cortical lymphocytes into the blood or lymph where they could then be subjected to ECIB or ECIL. The enhanced lymphocyte depletion produced by a combination of *B. pertussis* and ECIB/ECIL could be an adjunct for immunosuppression or possibly a treatment of lymphocytic leukemia. The toxic effects of *B. pertussis,* such as those due to histamine-sensitizing factor (HSF), hypoglycemia, and lethality (6, 7) pose serious limitations to its potential clinical use. In this report the results of efforts to separate the lymphocytosis-producing factor (LPF) from other toxic factors are presented.

Materials and Methods. Five-weeks-old Swiss albino (BNL strain) female mice, weighing 15–20 g, were used.

Supernatant fluid of *B. pertussis* cultures were kindly provided by Dr. S. I. Morse of the Rockefeller University, New York. This material had been prepared as follows: organisms of *B. pertussis* were cultured in liquid media (8) for 3–4 days to obtain 1–2 $\times$ 10^{10} cells/ml, then merthiolated, filtered, and centrifuged at 900*g* for 45 min. The clear, "pertussis supernate" (PS) was removed and stored at 4°. This PS was shown to be potent in inducing leukocytosis and lymphocytosis in mice (8).

The details of various chromatographic fractionations performed in these studies are shown in Table I. Sephadex was obtained from Pharmacia, Uppsala, Sweden and Bio Gels from Cal Biochem, Spring Valley, New York.

The molecular weight of the LPF was approximated from its elution volume on a calibrated Bio Gel, P-100 column. References were made to cytochrome C and egg albumin in appropriate concentration.

Bioassay of LPF was performed as follows: The PS was diluted to 1:2, 1:4, 1:8, and 1:16 with 0.05 *M* Tris buffer. The inocula were 0.2 ml, given intravenously via the tail vein and the mice were bled from the orbital venous plexus under light ether anesthesia on days 2, 4, and 6 or 7. Total leukocytes were counted in a Coulter counter, (Coulter Electronics, Hialeah, Fla.) Model A, with an orifice of 100 μ. Smears were stained with Wright and Giemsa and 200-cell differential counts were performed.

Blood glucose was determined by an auto-

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TABLE I. Column Chromatography of Supernatant Fluid of *B. pertussis* Culture (PS).

Series	Source	Sample vol (ml)	Column[b]	Vol/fraction (ml)	Maximum LPF[a] activity seen in fraction #	No. of mice tested
B	PS	15	Sephadex G-10[c]	15	4	150
C	PS	15	Sephadex G-10[c]	10	6	190
D	Fraction #6 from C (above)	10	Bio Gel P-100[d]	5	11	295
E	PS[e]	25	Bio Gel P-100[d]	2	20	85
F	PS	10	Bio Gel P-100[d] at 4°	2	24	105

[a] Lymphocytosis-producing factor.
[b] Eluent: 0.5 *M* NaCl in 0.05 *M* Tris buffer, pH 7.5.
[c] 1.5 × 80.5 cm.
[d] 1.5 × 78.5 cm, 50–100 mesh.
[e] Dialyzed against distilled water for 2 days, then evaporated to dryness; reconstituted in 3 ml of buffer containing 8% sucrose.

matic analyzer (Technicon Corporation, Tarrytown, N.Y.)

For the determination of HSF, 0.275 mg of histamine phosphate, equivalent to 0.1 mg of histamine (E. Lilly & Co., Indianapolis, Indiana) was injected intraperitoneally on day 4 following the injection of PS or its fraction.

Body weight was measured on days 0, 2, 4, and 6 or 7. The ratio of day 6 or 7 body weight to that of day 0 body weight was designated as body weight index. The animals were sacrificed on day 7, and the thymuses were weighed. The ratio of thymus weight to the body weight × 1000 was recorded as the thymus index.

Results. The influence of 0.2 ml of PS on body weight, mortality, histamine sensitivity, blood lymphocyte count, and blood sugar level are shown in Table II. When the PS in serial dilution up to 1:16 was used, it was observed (146 mice) that the lymphocytosis,

TABLE II. Biological Activities of the Various Chromatographic Fractions of Supernatant Fluid of *B. pertussis* Culture (PS).

Inoculum[a]	Dose (ml)	Body wt index[b]	Day 7 mortality	Histamine sensitivity		Total blood lymphocytes day 4 (mm³)	Blood glucose day 7 (mg/100 ml)
				Dose[c]	*D/T*[d]		
Buffer	0.2	1.071	0/10	0.2	0/11	9480	84.1
PS	0.2	0.929	3/10	0.1	5/5	56,000	57.6
B-4	0.2	1.062	2/10		NT[e]	51,690	NT
C-6	0.2	0.882	1/10		NT	67,200	NT
D-11	0.2	1.064	0/5	0.2	1/3	46,300	110.3
E-20	0.2	0.930	1/4		NT	33,570	113.5
E-20	0.1	NT	0/5	0.1	2/5	32,050	105.3
F-24	0.2	1.032	2/5	0.1	0/3	56,430	93.5
F-25	0.2	1.091	0/5	0.1	1/3	47,450	92.0
F-30	0.2	1.127	0/5	0.1	1/3	43,240	72.0
F-34	0.2	1.117	0/5	0.1	1/3	8660	120.6

[a] See Table I for details of identity of various fractions.
[b] The ratio of day-6 or day-7 body weight to day-0 body weight.
[c] Histamine (mg) injected on day 4.
[d] *D/T* = deaths/number of mice tested.
[e] NT = not tested.

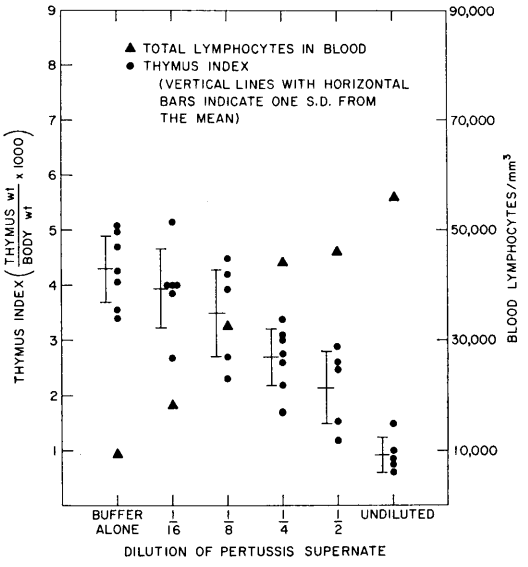

FIG. 1. Dose–effect relationship of supernatant fluid of *B. pertussis* culture on thymus weight and blood lymphocyte count in mice. Dose, 0.2 ml iv, of the supernate or its serial dilution from 1:2 to 1:16 in Tris buffer with control of buffer alone. Lymphocyte count: day 4; thymic index: day 7.

thymus weight loss (Fig. 1), body weight loss, mortality, and histamine sensitivity were dose dependent.

Sephadex G-10 column fractionation (Series B and C, Fig. 2) showed the LPF activity to be confined in a narrow zone. Although the lymphocytosis thus induced by fraction B-4 and C-6 was comparable to the effect of PS, these fractions were not free from the lethal factor (Table II).

When fraction C-6 (Fig. 2) was further fractionated on Bio Gel P-100 (Series D, Fig. 3) the LPF was isolated in fraction no. 11. This fraction, although free from the lethal factor, showed a slight degree of HSF activity but no hypoglycemia or weight loss (Table II).

In Series E, in which a dialyzed, evaporated concentrate of the PS was chromatographed, maximal lymphocytosis was produced by fraction no. 20. The lymphocytosis was the lowest as compared to the results from all other fractionations, and this fraction retained HSF, the lethal factor and caused some weight loss although no hypoglycemia was induced (Table II).

The series F chromatography was carried out at 4°. Fraction no. 24-induced lymphocytosis was comparable to that produced by the PS, and was predominantly due to an increase in the small lymphocytes (Fig. 4). This active fraction (no. 24) showed no HSF and hypoglycemia production, but killed some mice (Table II). In contrast, fraction nos. 25 and 30 showed a reversal of these two toxic factors (no lethality, but some HSF), with significant lymphocytosis. Fraction no. 34 had no LPF and no mortality but retained some HSF.

The molecular weight of the LPF as estimated from its retention volume on the Bio Gel P-100 column was 32,000. The chromatographic separations were monitored continuously at a wavelength of 280 mμ. Only a small portion of the total absorbing material was included in the active fractions.

Discussion. Clausen *et al.* (6), using anti-lymphocyte serum, demonstrated that the

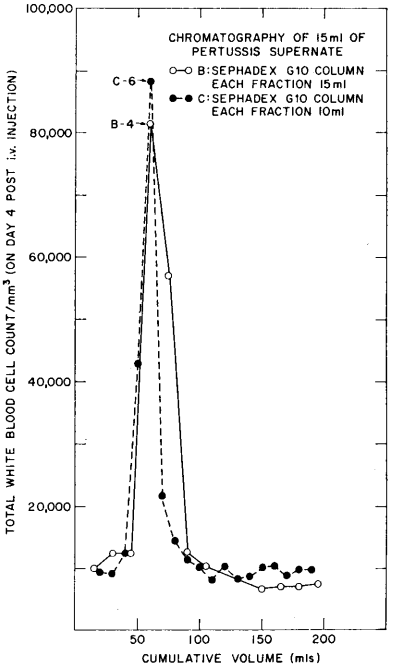

FIG. 2. Sephadex G-10 column chromatographic fractionation of 15 ml of supernatant fluid of *B. pertussis* culture. Series B and Series C: 15 and 10 ml, respectively, per fraction. The figure shows blood leukocyte count on day-4 post injection iv of 0.2-ml aliquot of each fraction in mice.

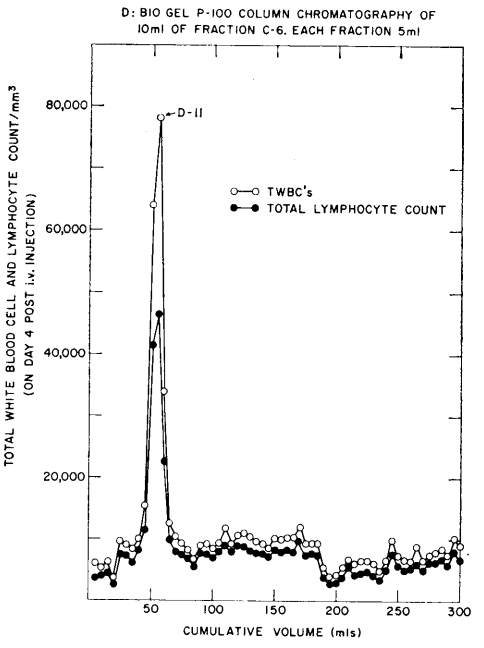

FIG. 3. Series D: Bio Gel P-100 column chromatographic fractionation of the 10-ml fraction no. 6 of series C. The figure shows blood leukocyte and lymphocyte counts on day-4 post injection iv of 0.2-ml aliquot of each fraction in mice.

lymphocytosis, increased histamine sensitivity and hypoglycemia in mice treated with cell extracts of *B. pertussis* were three phenomena independent of each other, but they did not try to separate these fractions individually. Bergman and Munoz (7) recently reported further observations confirming this conclusion using an alkaline saline extract of *B. pertussis*. Morse and Bray (8) were successful in isolating a water-insoluble fraction from the *B. pertussis* culture supernatant fluid which had more than 50-fold LPF activity than the intact bacteria. The results of their studies showed LPF and HSF to be closely associated and the attempts to separate these two factors from the supernatant fluid were unsuccessful. We now report a partial success in separating LPF from HSF, hypoglycemia-inducing factor, and the lethal factor in the *B. pertussis* culture supernatant. Using a combination of Sephadex G 10 and Bio Gel P 100 column chromatography, we obtained a fraction which retained its LPF but lost lethal and hypoglycemia-producing factors and partially lost HSF. It is of interest to note that when fraction C-6 was further fractionated, the resultant active fraction D-11 did not cause the body weight loss that was produced by the original C-6 (Table II).

When a Bio Gel P 100 column separation was carried out at 4°, we obtained a fraction which had retained LPF but had lost HSF. However, this fraction was not free from lethal effect. Some other fractions of this separation at 4° showed retention of LPF, no mortality and some reduction in HSF. None of the LPF active fractions chromatographed at 4° showed hypoglycemic activity. There has been considerable debate regarding the interrelationship between hypoglycemic and histamine-sensitizing effects of *B. pertussis* (7). Bergman and Munoz showed that hypoglycemia *per se* does not seem to explain the histamine sensitivity following *B. pertussis*

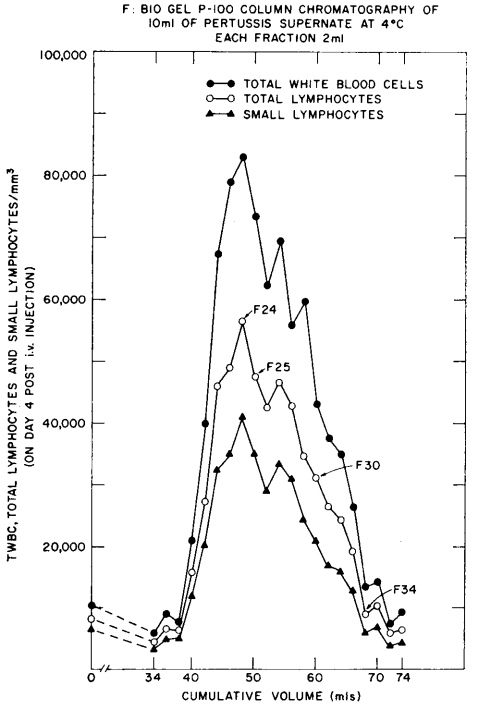

FIG. 4. Series F: Fractionation of 10 ml of supernatant fluid of *B. pertussis* culture at 4°. The figure shows blood leukocytes, total lymphocytes, and small lymphocytes on day-4 post injection iv of 0.2-ml aliquot of each fraction in mice.

treatment. Our success in separating LFP active fractions which retained HSF but lost hypoglycemic effect lends support to their results. Our failure in obtaining an active concentrate of the supernatant fluid may be ascribed to denaturation during dialysis and evaporation at room temperature (8).

Morse and Bray (8) noticed a maximum lymphocytosis with supernatant fluid of *B. pertussis* cultures at a dilution of 1:2, which, however, was not significantly different from that of undiluted culture. They ascribed this phenomenon to the presence of an inhibitor in the undiluted fluid. The results of our studies do not support this hypothesis, as we obtained a maximum lymphocytosis response with undiluted culture fluid (Fig. 1).

The thymic weight loss observed by Morse (9) and confirmed by us can be due to mobilization of thymic lymphocytes or their intrathymic destruction. This picture of peripheral lymphocytosis with thymic weight loss may be the result of alterations in the traffic patterns rather than the intrathymic destruction of lymphocytes. Such an hypothesis would be compatible with the results obtained by Kalpaktsoglou *et al.* (10) in normal and thymectomized mice treated with *B. pertussis.*

Summary. The *B. pertussis* culture supernatant fluid possessing lymphocytosis-producing factor, histamine-sensitizing factor, lethal and hypoglycemia-producing factors was subjected to Sephadex G-10 and Bio Gel P-100 column chromatography. When chromatographic fractionation was carried out at 4° some fractions, found to retain the lymphocytosis-producing property, had lost the lethal and hypoglycemia producing factors, and had partially lost the histamine-sensitizing factor. Observations indicate that lymphocytosis-producing factor is distinct from other toxic factors.

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