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Research Article

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ABSTRACT The purified lymphocytosis promoting factor (LPF) from *Bordetella pertussis* was found to be a potent mitogen for peripheral blood lymphocytes (PBL) from normal adults as well as for cord blood lymphocytes. Proliferation occurred in autologous plasma or fetal calf serum, regardless of previous exposure to pertussis infection or immunization. Only one adult human serum, from a physician constantly working with *B. pertussis*, inhibited the mitogenic response to LPF and this serum was shown to contain precipitating antibody against LPF. The proliferative effect of LPF was characteristic of a "nonspecific" mitogen and not of antigen stimulation of sensitized cells.

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INTRODUCTION

The association of peripheral blood lymphocytosis with clinical pertussis (whooping cough) has long been known (1). Both intact *Bordetella pertussis* organisms and culture supernatant fluids induce lymphocytosis in mice and many other experimental animals (2). Recently, the lymphocytosis promoting factor (LPF)[1] has been purified from *B. pertussis* culture supernatant fluids (3). In addition to its in vivo effects, LPF was found by Kong and Morse (4, 5) to be a potent mitogen for murine lymphocytes in vitro. LPF stimulated cells from lymph nodes and spleen but not from the thymus or bone marrow. The mitogenic response of murine lymphocytes to LPF was the result of activation of unsensitized T cells, but there was a requirement for an accessory nonphagocytic adherent cell (6). Furthermore, the subpopulation T cell responding to LPF was different from that stimulated by phytohemagglutinin (PHA) or concanavalin A (Con A).

The present studies were initiated to determine whether LPF was mitogenic for normal and abnormal human peripheral blood lymphocytes (PBL), and to identify the responding lymphocyte population.

[1] *Abbreviations used in this paper*: C, complement; CLL, chronic lymphatic leukemia; Con A, concanavalin A; E, sheep cell rosettes; EAC, sheep cell rosettes coated with antibody and complement; E_m, mouse cell rosettes; FCS, fetal calf serum; LPF, lymphocytosis promoting factor; LSA, lymphosarcoma; LSCL, lymphosarcoma cell leukemia; MRBC, mouse red blood cells; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; SRBC, sheep red blood cells.

METHODS

Subjects

Normal donors included 23 employees of the Columbia Presbyterian Medical Center and Downstate Medical Center. 23 patients from the Hematology Service at Harlem Hospital and Columbia Presbyterian Medical Center with lymphoproliferative disorders were also studied. Included were 13 patients (see Table III) with chronic lymphatic leukemia (CLL), 5 with lymphosarcoma cell leukemia (LSCL), and 5 with lymphosarcoma (LSA). PBL from two patients with Sezary syndrome were also tested.

Lymphocyte cultures

PBL were isolated from heparinized venous blood by a modification of the method of Boyum (7). 40–50 ml of blood were obtained from normal individuals in phenol-free heparin and allowed to sediment at 37°C for 1–2 h. The plasma was expressed from the top of the syringe and gently mixed with an equal volume of sterile isotonic saline. 2 vol of the saline-plasma solution were layered over 1 vol of Ficoll-Conray solution (10 ml 33.4% Conray, meglumine iothalamate, prepared from a 60% aqueous solution + 24 ml 9% Ficoll) and centrifuged for 30 min at 4°C (Ficoll was from Pharmacia Fine Chemicals, Piscataway, N. J., and 60% Conray was from Mallinckrodt Inc., St. Louis, Mo.). The interface was removed and washed three times with RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Usually 85% of the cells were lymphocytes and 15% monocytes. The trypan blue dye viability was >95%. The cells were cultured in this medium with 5% fetal calf serum (FCS; Grand Island Biological Co.), 10% pooled human AB plasma, or 10% autologous plasma. Except for autologous systems, all sera and plasma were heated to 56°C for 30 min before use.

Triplicate cultures of 0.25 ml containing 1 or 2×10^5 lymphocytes per well were placed in microtiter plates (Linbro IS FB96, Linbro Chemical Co., New Haven, Conn.), and incubated at 37°C in 5% CO_2:95% air for various time periods. The cultures were then pulsed overnight with 1 μCi/well of [^{3}H]thymidine, 6.7 Ci/mmol (New England Nuclear, Boston, Mass.). The microtiter cultures were harvested and washed extensively with saline on glass fiber filters (grade 934 AH, Reeve Angel, Clifton, N. J.) using a Mash II Harvester (Microbiological Associates, Bethesda, Md.; 8). The filters were dried and placed in 12 ml of Instabray (Yorktown Research Inc., S. Hackensack, N. J.). ^{3}H content was determined in a liquid scintillation spectrometer (Packard model 3320, Packard Instrument Co., Inc., Downers Grove, Ill.). The results are expressed as mean cpm of triplicate samples.

Mitogens

The mitogens used included LPF, PHA-P and Con A. LPF was purified by methods previously described (3). Because it is kept in urea, the LPF controls initially contained the exact amount of urea for all LPF concentrations, but inasmuch as these controls showed similar counting rates to the resting unstimulated cultures, the later experiments used only the amount of urea corresponding to the highest concentrations of LPF. All preparations of LPF were tested for mitogenicity on murine lymphocytes before use on human PBL.

Dose-response curves with PHA-P and Con A were performed in a previous study (9), and all normal individuals responded to the two doses of each mitogen selected to serve as positive controls for mitogen lymphocyte stimulation. Peak stimulation with both mitogens occurred at 5 days. The cultures contained either 0.001 or 0.005 ml stock/ml PHA-P (Difco Laboratories, Detroit, Mich.) or 1, 5 and occasionally 50 μg/ml Con A (Calbiochem, San Diego, Calif.).

Lymphocyte rosette techniques

Sheep red blood cell (SRBC) rosettes (E). A modification of the method of Wybran et al. (10) was used to obtain lymphocytes with receptors for SRBC. Equal volumes of a 2×10^6/ml lymphocyte suspension were incubated for 15 min at 37°C with 0.5% SRBC (the SRBC had been stored in Alsever's solution for 1–3 wk). After centrifuging at 200 *g* the pellet was gently overlaid with 0.2–0.4 ml of FCS, which had been absorbed against SRBC before use, and incubated at 4°C for >1 h. The pellet was gently resuspended and an aliquot removed to count the percent of SRBC (+) rosettes. A rosette was defined as a lymphocyte with three or more adherent SRBCs. The remaining rosettes were placed on a Ficoll-Conray gradient, centrifuged, and the heavy rosetted lymphocytes and those left at the interface were harvested separately. After washing and lysing the SRBC, the cell concentrations were equalized and in some experiments reconstituted in varying ratios of E (+) to E (−) cells.

Antigen-antibody-complement complex (EAC) rosettes. The method of Bianco et al. (11) was used to obtain lymphocytes with receptors for mouse complement (C). Fresh mouse serum (C) was obtained from the Pocono Rabbit Farm (Canadensis, Pa.) and 19S rabbit anti-sheep cell antibody from Cordis Laboratories (Miami, Fla.). Equal volumes of 5% SRBC suspension and a 1:100 dilution of anti-sheep cell antibody in saline (maximum nonagglutinating dose) were incubated for 15 min at 37°C in a water bath, to make EA. The EA were washed, centrifuged, and resuspended to the intitial volume of E in veronal buffer; reserving an aliquot for appropriate controls. Equal volumes of EA and a 1:10 dilution of mouse C were incubated for 45 min at 37°C. After washing, the EAC were resuspended to the initial volume with RPMI. Equal volumes of 2×10^6/ml lymphocytes and a 0.5% suspension of EAC were incubated at 37°C for 30 min with gentle shaking. After washing, the percentage of rosettes was counted and the cells processed at 22°C for gradient centrifugation as for the E rosettes.

Mouse cell rosettes (E_m). Lymphocytes with receptors for mouse red blood cells (MRBC) were isolated by the method of Gupta and Grieco (12). Equal volumes of a 1×10^7/ml lymphocyte suspension and heated FCS which had been absorbed with MRBC before use were added to each other and then added to an equal volume of 2% MRBC. The mixture was centrifuged for 5 min at 22°C and 150 *g*, followed by incubation at 22°C for 1 h. The pellet was gently resuspended and the percentage of rosettes determined before separation by gradient centrifugation.

RESULTS

Mitogenic effect of LPF on normal human PBL. In the initial experiments a wide range of doses of LPF were tested for the ability to stimulate normal human PBL obtained by Ficoll-Conray density separation. A representative dose-response curve is illustrated in Fig. 1. In all instances, stimulation of [^{3}H]thymidine incorporation occurred only in a very narrow dose

range of LPF. Maximum stimulation usually occurred between 2 and 4 μg of LPF/ml and there was no response to concentrations below 0.1 or above 8 μg/ml. In Fig. 1, it can also be seen that LPF produced comparable or even greater stimulation of normal PBL when compared with the responses to two concentrations of PHA-P and Con A.

These results are illustrated more extensively in Table I which shows the level of lymphocyte stimulation by LPF and PHA-P using PBL from 19 normal adult individuals over 20 yr of age. In initial experiments 10–15% autologous plasma was used, and it was anticipated that antipertussis antibody resulting from immunization with DPT or childhood pertussis (whooping cough) might inhibit LPF stimulation. The upper section of Table I shows the results with the PBL of seven individuals which were cultured in autologous plasma. Two had no known immunization or clinical history of pertussis; three had a childhood history compatible with pertussis; and two received childhood DPT immunization. All PBL had a full response to LPF in autologous plasma except those from subject S.I.M. As shown in Fig. 2, the PBL from subject S.I.M. responded normally to PHA in both AB and autologous plasma and to LPF in AB, but not autologous plasma. PBL from S.I.M. also responded in FCS. S.I.M. has worked extensively with *B. pertussis* and is known to have anti-LPF antibody (Fig. 3). A surprising finding was the partial inhibition of the Con A response of PBL cultured in S.I.M. plasma. This cross-inhibition is presently under study.

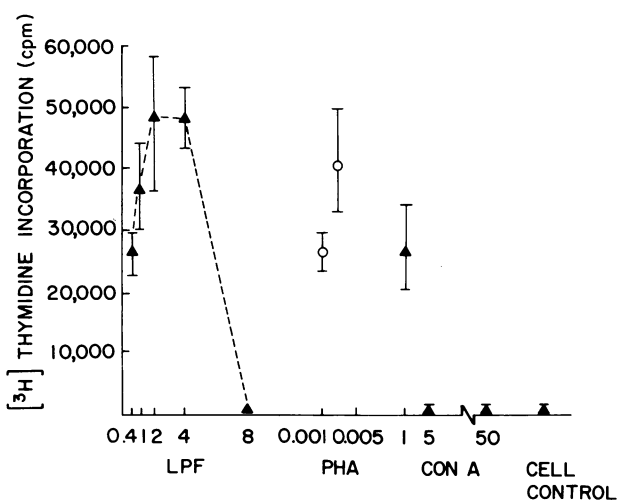

FIGURE 1 Effect of LPF, PHA-P, and Con A on human PBL. Each culture, performed in triplicate, contained 1×10^5 lymphocytes in 0.25 ml culture medium with 5% FCS and was incubated for 5 days. Results are expressed as the arithmetic mean ± 1 SD of cpm of [^{3}H]thymidine incorporation per culture. The concentration of LPF is expressed as μg/ml; PHA as ml/ml; and Con A as μg/ml of culture.

TABLE I
Effect of LPF and PHA on PBL from Normal Adults

Subject	Exposure to pertussis	[^{3}H]Thymidine incorporation* LPF	PHA-P	Unstimulated
Cultured in autologous plasma				
1. E. B. (2)	None	11,421±1,375	15,287±79	116±4
E. B.		35,571±2,388	4,458±614	81±1
2. P. D.	None	56,680±9,426	ND	54±9
3. J. H. M.	Pertussis	28,617±3,517	20,953±1,023	105±11
J. H. M.		59,652±5,474	ND	82±35
J. H. M.		51,432±8,541	49,282±3,095	106±9
4. S. I. M.	Pertussis	1,216±304	39,954±6,336	213±47
5. H. N.	Pertussis	53,796±2,203	ND	149±52
6. G. O.	DPT vaccine	48,852±2,399	43,841±8,071	234±71
G. O.		81,361±7,149	ND	334±19
7. A. P.	DPT vaccine	30,262±714	57,473±7,773	293±91
Cultured in FCS				
8. M. D.		21,545±1,082	26,829±6,957	379±120
9. D. E.		43,990±1,211	17,925±1,063	252±51
10. D. F.		30,907±6,033	20,631±2,117	99±5
D. F.		33,331±3,297	ND	
11. K. F.		8,874±352	20,976±2,470	193±7
12. E. L.		38,224±1,297	37,748±2,892	288±16
13. K. M.		16,902±2,091	3,848±343	67±1
K. M.		38,075±1,657	34,998±3,344	153±11
14. P. C.		48,905±8,005	40,627±5,952	139±27
15. C. C.		37,887±7,357	23,395±3,278	154±48
16. M. P.		43,353±3,208	40,036±2,525	116±4
17. L. S.		3,183±374	8,590±1,571	ND
L. S.		3,338±546	13,147±1,449	71±4
18. L. Sch.		26,199±7,601	50,410±6,987	137±12
19. T. V.		21,225±2,445	24,811±3,586	107±8

* Results are expressed as the arithmetic mean±SE. Culture time was 5–7 days except for E. B. (2) which was 2 days.

For subsequent experiments, FCS was employed in order to minize any contribution of inhibiting factors in human plasma. As shown in Table I, PBL from 12 individuals gave good mitogenic responses to LPF and PHA-P when FCS was the serum supplement.

The time-course of LPF responsiveness was studied and maximum stimulation was found to occur after 5–7 days of incubation. Unless otherwise noted, this time period was used in all experiments. More than 10 purified preparations of LPF have been used to date and found to be potent mitogens for normal PBL. Fig. 4 shows that equivalent maximal stimulation was achieved by two different preparations of LPF on the same PBL. The concentration of LPF required for

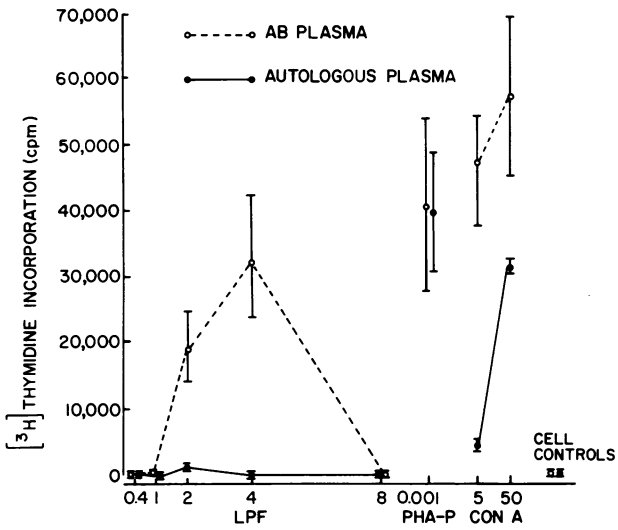

FIGURE 2 Comparison of LPF, PHA, and Con A stimulation of PBL in an autologous plasma containing anti-LPF antibody and in normal AB plasma. The concentration of LPF is expressed as μg/ml culture, PHA as ml/ml of culture, and Con A as μg/ml of culture. Cultures were supplemented with either 15% AB or 15% autologous plasma. Results are expressed as the arithmetic mean ± 1 SD of cpm of [^{3}H]thymidine incorporation per culture.

maximum stimulation was greater in the older preparation of LPF which is consonant with the finding in the murine system that LPF loses its mitogenicity upon prolonged storage, presumably as a result of aggregation.

The fact that PBL from all normal adults tested responded to LPF in antibody-free medium suggested that LPF, although derived from an organism with which most individuals have had contact, either as immunogen or pathogen, was not causing lymphocyte proliferation on the basis of antigen stimulation of previously sensitized lymphocytes. Moreover, with the exception of S.I.M., no serological evidence of prior LPF exposure could be found. Nevertheless, in order to rigorously exclude the possibility that sensitized cells were the responders, cord blood lymphocytes were tested for stimulation by LPF. As shown in Table II, samples of cord blood cells responded to LPF as well as to Con A and PHA-P, again suggesting that LPF was acting as a "nonspecific" mitogen rather than as an antigen. The maximum response to each mitogen varied among the three cord blood lymphocytes tested, suggesting that distinct cell populations might be preferentially stimulated.

Mitogenic effect of LPF on normal PBL separated by E, EAC, and MRBC rosetting techniques. The next series of experiments was designed to determine the subpopulation of normal lymphocytes responding to LPF. First, normal PBL obtained by gradient separation were separated by the SRBC technique. The response of unseparated PBL to LPF and PHA was compared to that found with (*a*) PBL with receptors for SRBC [E(+), T enriched]; (*b*) PBL remaining after rosetting with SRBC [E(−), T depleted]; and (*c*) a mixture of 80% E(+) and 20% E(−) cells. The cell concentrations were the same in the four groups.

The results of a representative experiment are shown in Fig. 5. It can be seen that neither E(+) cells nor E(−) cells alone were capable of responding optimally to LPF when compared to the stimulation obtained with unseparated cells. In contrast, the reconstituted population of 80% E(+) and 20% E(−) cells reacted normally. The E(+) population gave 20,000 cpm or approximately one-third of the maximum LPF stimulation of 60,000 cpm, whereas the E(−) cells gave 5,000 cpm or only about one-twelfth of maximal stimulation. In contrast to LPF, PHA proved fully mitogenic for the E(+) cells as well as for the unseparated cells and the reconstituted cells, but like LPF was only minimally mitogenic for the E(−) cells.

Individual variations occurred with the PBL from four normal individuals examined in this manner, but the inability of the isolated SRBC receptor-positive

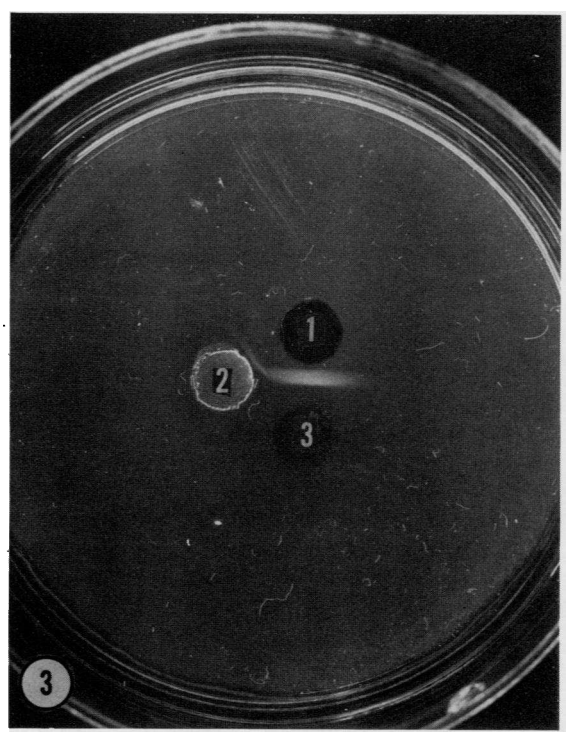

FIGURE 3 Gel diffusion analysis (Ouchterlony) of purified LPF with an antibody containing Na_2SO_4 precipitate of serum S.I.M. and rabbit hyperimmune antipertussis serum. Well no. 1 contains purified LPF, 200 μg/ml; well no. 2 a 20% $NaSO_4$ precipitate of serum S.I.M., concentrated five times; and well no. 3, hyperimmune rabbit anti-LPF antiserum.

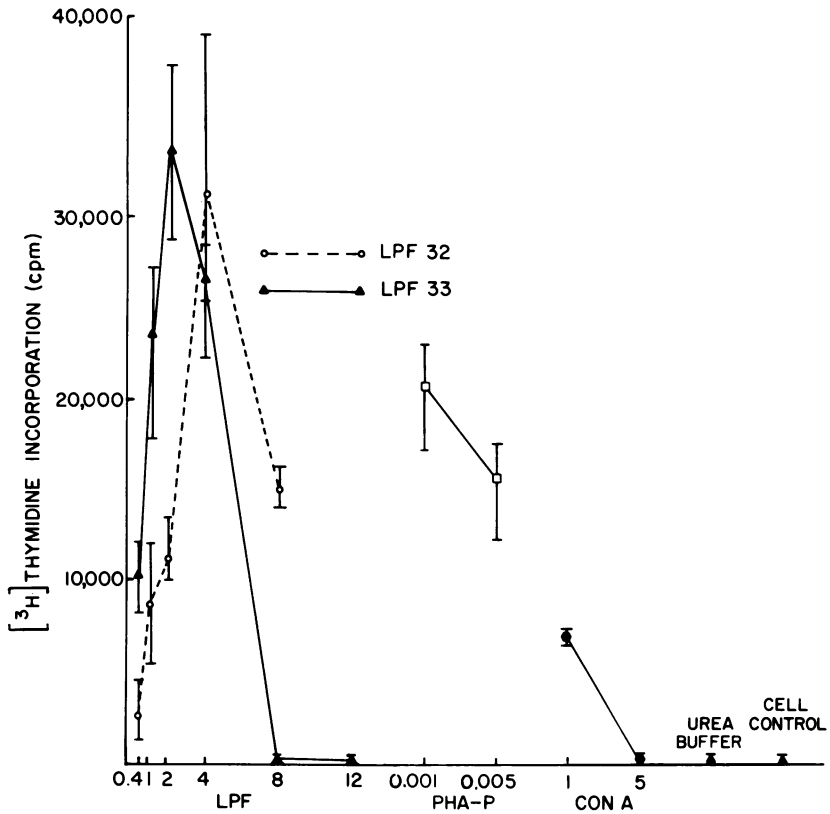

FIGURE 4 Comparison of the mitogenicity of two different preparations of LPF. LPF preparation no. 32 was more than 3 wk older than preparation no. 33 at the time of assay. LPF concentrations are expressed as μg/ml; PHA as ml/ml; and Con A as μg/ml of culture.

TABLE II

Effect of LPF, PHA-P, and Con A on Cord Blood Lymphocytes

		[3H]Thymidine incorporation (cpm)						
		LPF			Con A		PHA-P	
Subject	None	1.5 μg	1.0 μg	0.5 μg	12.5 μg	1.25 μg	1.25 μg	2.5 μg
Cord blood no. 1	34,859 ±7,243	172,424 ±3,836	127,960 ±6,650	175,464 ±26,814	4,794 ±294	110,237 ±8,980	224,947 ±12,672	196,760 ±9,030
Cord blood no. 2 (72 h)	5,707 ±955	41,228 ±1,179	170,531 ±11,985	236,168 ±12,157	19,128 ±467	151,789 ±1,618	65,620 ±1,735	38,117 ±1,837
(120 h)	35,379 ±4,045	18,669 ±241	216,054 ±11,583	88,376 ±4,363	830 ±168	73,799 ±11,830	249,218 ±17,381	114,653 ±2,230
Cord blood no. 3	18,370 ±6,303	113,910 ±15,063	84,948 ±19,841	46,350 ±3,719	4,311 ±394	58,785 ±2,590	115,153 ±3,198	48,998 ±2,409
Normal adult no. 1	844 ±200	104,855 ±13,774	246,853 ±12,972	189,274 ±18,821	761 ±68	147,487 ±8,256	221,081 ±22,352	146,939 ±19,910
Normal adult no. 2	1,255 ±1,047	4,254 ±473	68,203 ±7,619	316,381 ±19,678	505 ±349	159,023 ±30,596	241,600 ±7,842	160,223 ±8,560

Results are expressed as the arithmetic mean±1 SD of cpm of [3H]thymidine incorporation per culture. Culture period was 120 h. Cord blood no. 2 was harvested also at 72 h.

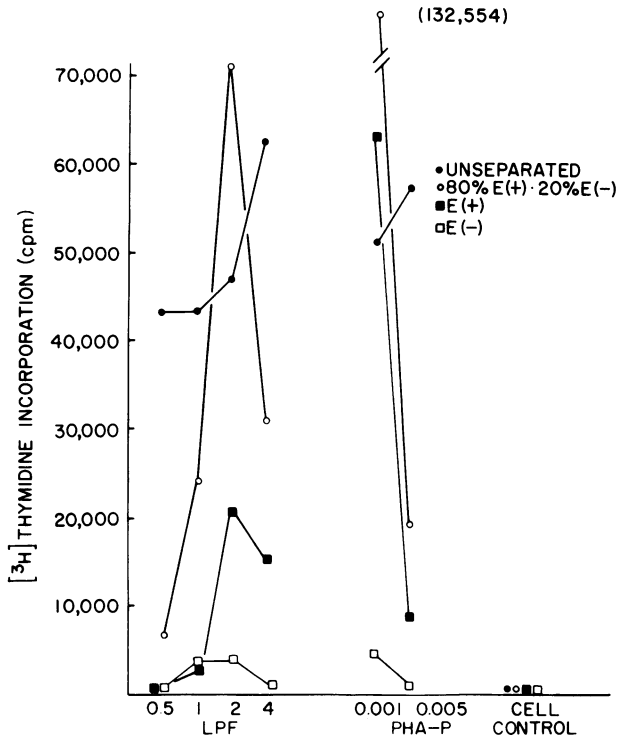

FIGURE 5 Effect of LPF and PHA on normal PBL separated by the SRBC rosette technique (E). PBL were separated into SRBC positive, E(+), and SRBC negative E(−) populations as well as recombined in a ratio of 80% E(+) and 20% E(−). The cell concentrations for all four groups were 5×10^5/ml. The initial ratio of the unseparated cells was 60% E(+) and 40% E(−).

cells to respond to LPF was consistent, whereas there was a full PHA effect.

PBL were next separated on the basis of the presence, EAC(+), or absence, EAC(−), of receptors for mouse C. SRBCs were coated with 19S rabbit anti-SRBC antibody and mouse C by the technique of Bianco et al. (12), and the rosettes placed on Ficoll-Conray density gradient. This technique selected out B lymphocytes with C receptors, EAC(+), leaving behind monocytes and all the lymphocytes lacking C receptors, EAC(−). Fig. 6 shows a representative experiment comparing the mitogenicity of LPF and PHA for PBL from an individual with 14% EAC(+) rosettes before separation. The unseparated cells and the EAC(−) population gave almost equivalent maximal responses to LPF. The EAC(+) cells gave less than half maximal stimulation to LPF as did the cells recombined in the ratio of 80% EAC(−):20% EAC(+) cells. However, maximal PHA stimulation was achieved with all four groups. The data again suggest that a different cell population(s) is required for LPF mitogenicity as opposed to PHA.

In the third mode of separation, cells with receptors

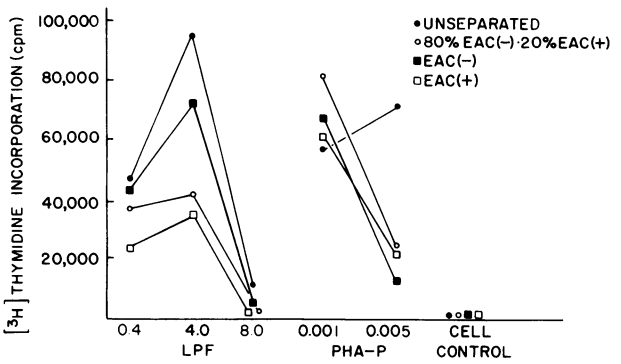

FIGURE 6 Effect of LPF and PHA on normal PBL separated by rosetting techniques with EAC. PBL were separated into those with receptors for mouse C using EAC(+), and those lacking these receptors, EAC(−). These two populations were also recombined in an 80% EAC(−) and 20% EAC(+) ratio. The cell concentration in all four groups was 8×10^5/ml. The initial ratio of the unseparated cells was 86% EAC(−) and 14% EAC(+). Although not plotted, a 60% EAC(−) and 40% EAC(+) ratio produced similar [^{3}H]thymidine incorporation to the 80% EAC(−) and 20% EAC(+) ratio.

for mouse erythrocytes were isolated by the rosetting technique. This method selects for lymphocytes containing surface immunoglobulin (11). In the experiment illustrated in Fig. 7, the unseparated cells gave the best response to LPF, whereas those cells with receptors for MRBCs, $E_m(+)$, were unresponsive. The combination of 80% $E_m(-)$:20% $E_m(+)$ as well

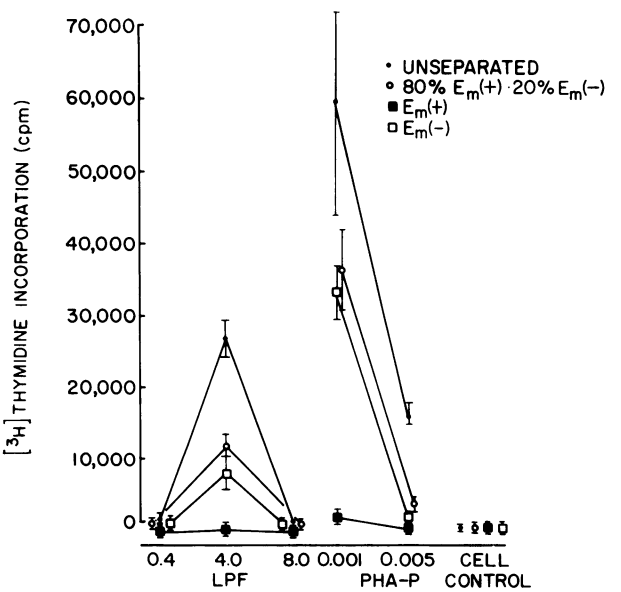

FIGURE 7 Effect of LPF and PHA on normal PBL separated by the MRBC rosetting technique. PBL were separated into $E_m(+)$ and $E_m(-)$ populations as well as recombined in a ratio of 80% $E_m(-)$ and 20% $E_m(+)$. The cell concentration for all four groups was 8×10^5/ml; the initial ratio of the unseparated cells was 89% $E_m(-)$ and 11% $E_m(+)$.

as the $E_m(-)$ population alone gave approximately one-third the maximal response. As in the case of E(+) and E(−) cells, neither isolated population was capable of responding to LPF, suggesting that a complex interaction of cells was required for mitogenicity. Fairly good mitogenic responses were obtained with PHA in all groups except for those cells with receptors for MRBCs, which are presumably B lymphocytes containing surface immunoglobulin.

Mitogenic effect of LPF on PBL from patients with chronic lymphatic leukemia (CLL), lymphosarcoma cell leukemia (LSCL), and lymphosarcoma (LSA). PBL from 23 patients with peripheral blood lymphocytosis associated with CLL, LSCL, and LSA were isolated by gradient centrifugation by the same method used for obtaining normal PBL and then cultured in FCS. These 23 patients were divided into three clinical groups illustrated in Table III. 13 had CLL, 5 LSCL, and 5 LSA.

Groups I and II had CLL and chronic LSCL as defined morphologically and clinically by Zacharski and Linman (13). In four of the patients in group III (Table III), the illness began as a disorder presenting with lymph node enlargement (biopsy proved as lymphocytic LSA) without increased numbers of circulating lymphocytes and later progressed to a phase in which increasing numbers of abnormal lymphocytes were found in the peripheral blood (14). In case 21, the illness initially presented with increased numbers of LSA cells in the peripheral blood without organ involvement (stage 0 according to the classification of Rai et al. (15). At the time of study 23 yr later, after multiple courses of therapy with alkylating agents, the patient had minimal involvement of the peripheral blood with periodic massive enlargement of cervical and inguinal lymph nodes. The patients were placed in these three groups by one of us (J.L.) without knowledge of the results of mitogen studies. Note that most patients with CLL were not receiving therapy at the time of testing, unlike those with LSA.

Table IV illustrates the mitogenic responses of these same 23 leukemic patients to LPF and PHA-P as measured by [^{3}H]thymidine incorporation. A response was considered positive at counting rates of 1,000 cpm or greater which corresponded to a stimulation index of 5 or greater. Of the PBL from the 13 patients with CLL, only 2 responded to LPF and these same 2 as well as an additional 2 responded to PHA. One of the two LPF-responsive individuals became unresponsive to both mitogens upon retesting later in the course of the disease. Of five individuals with LSCL, only one responded to LPF, whereas all responded to PHA. One PHA-responsive patient later became unresponsive. Four of five individuals with LSA had normal or near normal responses to both mitogens. The one unresponsive patient (no. 23) in this group was unusual in that she had a histologic diagnosis of a nodular, well-differentiated lymphocytic LSA, whereas the other four had poorly differentiated lymphocytic LSAs.

Of the 18 patients with CLL or LSCL, only 3 responded to LPF whereas 9 responded to PHA. Thus PHA is more likely to give a mitogenic response in this group of B-cell leukemias, and LPF may be a more useful mitogen than PHA in identifying these patients. Both LPF and PHA were mitogenic for the PBL of LSA patients with poorly differentiated LSAs, however these patients were also on chemotherapy.

It can also be noted in Table IV that PBL from two of five patients in the LSA group had high [^{3}H]thymidine incorporation in the resting state. Because these cultures were performed in FCS, making the effect of a humoral factor unlikely, it is possible that some "normal" cells were proliferating in response to the "abnormal" leukemic cells. It is also possible that lack of control of normal autologous stimulation occurred, or that drug therapy in some way modified the normal response, or the leukemic cells were proliferating at a faster rate than normal. [^{3}H]thymidine incorporation in the unstimulated cultures using PBL from the CLL and LSCL group were low or normal. Four of the unresponsive individuals were retested at a later date with LPF to be sure of the validity of the negative response. Only one, who was receiving chemotherapy and was in remission, responded, suggesting that LPF unresponsiveness persists without chemotherapeutic intervention.

Two patients with Sezary syndrome were tested because this is a T-cell leukemia (16). One was unresponsive to both LPF and PHA with counting rates below 600 cpm, whereas the other responded weakly with maximal responses ranging between 1,400 and 1,900 cpm for both mitogens. Although other leukemias were not systematically investigated, one patient with chronic myelogenous leukemia responded normally to both mitogens.

DISCUSSION

The product of *Bordetella pertussis* which causes leukocytosis with a predominance of mature-appearing small lymphocytes has recently been isolated and characterized (3). The purified LPF was found to be a potent, nonspecific mitogen for mouse T lymphocytes (6).

The experiments performed in this study were designed to determine (*a*) whether LPF is a mitogen for human lymphocytes, (*b*) whether the mitogenic effect is related to prior exposure to antigen, (*c*) the comparative effects of LPF, PHA, and Con A on lymphocyte populations, and (*d*) the nature of the responding cells.

PBL from six of seven adults were stimulated by

TABLE III

Clinical Features of the Patients with CLL, LSCL, and LSA

Case	Patient	Age	Sex	Lymphocytes	Stage*	Enlarged			Anemia	Thrombocytopenia	Duration of disease	Therapy‡
						Nodes	Liver	Spleen				
				$\times 10^3/\mu l$							*yr*	
Group I CLL												
1.	L. A.	73	M	34.7	III	+	0	0	+	0	3	0
				28.4	III	+	0	0	+	0	3.3	pred
2.	M. D.	77	F	14.2	II	+	0	+	0	0	1	0
3.	L. F.	69	M	182.4	IV	+	+	+	+	+	5	chlor
4.	E. G.	77	F	78.2	III	0	0	0	+	0	5	0
5.	P. G.	69	M	75.0	IV	+	+	+	+	+	3	chlor§, pred§
6.	E. H.	75	F	73.5	III	+	0	0	+	0	1	0
7.	P. H.	69	M	31.2	III	+	+	+	+	0	10	0
8.	E. H.	84	F	16.5	0	0	0	0	0	0	1	0
9.	J. I.	64	M	30.4	IV	+	+	+	+	+	3	chlor, pred
10.	A. J.	78	F	250.0	IV	+	+	+	+	+	4	0
11.	J. L.	79	F	26.3	0	0	0	0	0	0	1	0
12.	J. O.	66	M	230.0	III	+	+	+	+	0	1	0
13.	T. W.	76	M	34.3	IV	+	0	+	+	+	12	chlor§
Group II Chronic LSCL												
14.	A. H.	75	F	26.9	0	0	0	0	0	0	7	0
15.	A. H.	77	M	14.6	IV	+	+	+	+	+	7	chlor§
16.	V. L.	61	M	122.6	0	0	0	0	0	0	7	0
				78.6	0	0	0	0	0	0	7.8	0
17.	S. P.	78	F	10.7	0	0	0	0	0	0	1	0
18.	H. P.	67	F	17.2	III	+	0	0	+	0	5	chlor
Group III LSA												
19.	J. L.	69	M	2.0	IV	+	+	+	+	0	2	chlor, pred, vcr
20.	G. N.	57	F	1.8	IV	+	+	+	+	+	1	chlor, pred, vcr
21.	M. P.	65	F	6.3	IV	+	+	+	+	+	23	chlor
22.	A. T.	67	F	20.0	IV	+	0	0	+	+	2.5	pred, splenectomy§
				22.8	IV	+	+	+	+	+	3	radio-rx
23.	O. B.	47	F	2.2	III	+	0	0	0	0	9	chlor

In group III histologic diagnosis of diffuse, poorly differentiated lymphocytic LSA was made on lymph node biopsy in patients 19–22; case 23 had nodular, well-differentiated lymphocytic LSA.

* Patients in groups I and II were staged according to the system of Rai et al. (15). Patients in group III were staged clinically according to the Ann Arbor Symposium criteria (15).

‡ pred = prednisone; chlor = chlorambucil; vcr = vincristine; radio-rx = radiotherapy. Patients were receiving therapy as listed at the time of the study except where indicated by§.

§ Therapy given more than 6 mo before study. Duration of disease as estimated from first detection of peripheral blood lymphocytosis or first symptom (whichever was earliest).

LPF when the cells were cultured in autologous plasma irrespective of a prior history of clinical pertussis or pertussis immunization (Table I). The PBL from the nonresponder, however, were stimulated by LPF when cultured in AB plasma or FCS. S.I.M. has worked with *B. pertussis* for many years and his serum is known to contain LPF antibody which inhibits proliferation. Despite a history of clinical whooping cough or immunization, the other plasmas did not inhibit suggesting that, like other pertussis antibodies, anti-LPF does not persist into adulthood after clinical infection or DPT immunization (17). However, there are no data as to whether anti-LPF antibody is produced normally in response to clinical infection or DPT immunization.

PBL from an additional 12 normal adults were cultured with FCS as the serum component of the medium, and all were stimulated by LPF. Thus, PBL from all

19 normal adult subjects responded to LPF, suggesting that stimulation by LPF was not a reflection of a response of cells previously sensitized to LPF or cross-reactive antigens. This was substantiated by the finding that cord blood cells responded fully. These results are consonant with the findings in the murine system where responsiveness of lymphocytes to LPF was also shown to be unrelated to presensitization to antigen (5). Although lymphocytosis often accompanies clinical pertussis, except in infants under 6 mo of age, blast cells are not found in the blood (1). It has been shown in experimental animals that the increase in ciruclating lymphocytes is the result of redistribution of cells and not to proliferation (18). The discrepancy between the in vivo and in vitro findings may be due to (*a*) the narrow dose range for in vitro stimulation of lymphocytes by LPF which might not be realized in vivo and/or (*b*) the fact that a much greater relative amount of LPF is required for mitogenicity in vitro than for production of lymphocytosis. It is possible, however, that lymphocyte proliferation occurs in tissues adjacent to the site of infection where a gradient might provide the appropriate concentration of LPF.

The purified LPF used in these experiments is a four-chain polypeptide with a molecular weight of approximately 74,000 and is free of carbohydrate and lipid (3). Thus, the LPF does not contain lipopolysaccharide endotoxin which might exert a proliferative effect. Furthermore, in contrast to endotoxin, LPF does not stimulate mouse B cells (6).

LPF, PHA, and Con A were roughly equivalent in potency, although variations occurred. LPF was often the most potent of the three mitogens for normal adult PBL, as well as for cord blood cells. Maximal stimulation of 4–8 × 10^5 cells/ml was obtained in most instances with 2–4 μg/ml of LPF, and the dose-response curve was very narrow in contradistinction to the broad dose-response curve of PHA. Mitogenicity with LPF rarely occurred below 0.1 or above 8.0 μg/ml. High dose inhibition did not appear to be the result of toxicity because the trypan blue dye exclusion test showed no excess cell death.

PBL from patients with the B-cell leukemias, CLL and LSCL, showed virtually no response to LPF, even less than that obtained with PHA. The poor response of these leukemic cells to mitogens, especially PHA, has been noted before and when there is a response it is usually due to nonleukemic cells (19). In the case of LSA where peripheral blood lymphocytosis includes T- and/or B-cell proliferation, PBL from four of five patients were responsive to both LPF and PHA. The PBL of two patients with Sezary syndrome, a leukemia of helper cell T cells (20), responded poorly to both LPF and PHA. The differential effect of LPF and PHA on PBL from patients with CLL and LSCL suggests that LPF may ultimately be more useful than PHA in defining these patients. The differences in the reactivity to these two mitogens may reflect stimulation of different lymphocyte populations.

TABLE IV
Effect of LPF and PHA on PBL from Patients with CLL, LSCL, and LSA

Subject	[^{3}H]Thymidine incorporation*			Days in culture
	LPF	PHA	Unstimulated	
Group I CLL				
1. L. A.	451±85	979±181	133±18	2
L. A.	11,357±2,679	24,562±3,564	205±58	7
L. A.	86±12	256±131	84±4	7
2. M. D.	116±12	6,391±964	105±12	6
3. L. F.	73±4	73±8	61±10	6
4. A. G.	156±25	525±91	103±1	7
5. P. G.	88±10	383±52	80±3	7
6. E. H.	107±35	112±48	70±8	7
7. P. H.	77±15	411±187	62±4	6
8. E. H.	17,487±1,348	15,354±1,944	106±6	7
9. J. I.	75±7	73±2	78±6	7
10. A. M. J.	146±72	482±186	147±61	7
11. J. L.	130±17	5,440±411	111±9	5
12. J. O.	95±2	264±50	100±10	6
13. T. W.	202±52	29±62	119±8	7
Group II LSCL				
14. A. H.	255±22	1,054±144	117±15	2
A. H.	146±28	640±95	117±9	7
15. A. HUN.	448±119	9,673±1,433	184±5	7
16. V. L.	89±19	10,675±596	64±4	5
V. L.	105±25	149±51	74±2	7
17. S. P.	134±10	2,891±659	114±3	7
18. H. P.	1,527±54	2,829±1,047	82±8	7
Group III LSA				
19. J. L.	16,388±979	3,779±122	146±58	5
20. G. N.	23,682±3,683	40,712±6,544	146±27	5
21. M. P.	34,961±7,115	13,659±1,591	658±201	7
22. A. T.	7,519±1,358	5,484±1,767	1,413±282	7
A. T.	2,784±159	4,852±396	3,001±663	7
23. O. B.	373±351	106±16	51±6	7

* Results are expressed as the arithmetic mean±1 SE of cpm of [^{3}H]thymidine incorporation per culture.

The lack of effect of LPF on PBL from patients with B-cell leukemias suggests that LPF is not a B-cell mitogen. PBL from normal adults were separated into T-cell enriched and B-cell enriched fractions using three different rosetting techniques and recovering the rosetting and nonrosetting fractions by gradient centrifugation. In each case the T-cell enriched fraction responded to a greater extent than the B-cell enriched fraction. This was particularly true of the experiments using E rosetting (Fig. 5), where the rosetting population is T-cell enriched, and E_m rosetting (Fig. 7), where the rosetting population is B-cell enriched.

However, in no instance did the isolated popula-

tion respond fully; confirmation of the hypothesis that T cells are stimulated by LPF is required in view of the finding that reactivity to LPF, unlike PHA, requires a multicomponent cellular system. The modes of cell separation used did not permit evaluation of the role of the monocyte. It is pertinent to note that the maximal response of mouse T cells to LPF also requires accessory cells which are not monocytes or B cells (21).

In summary, LPF is a potent mitogen for unsensitized human PBL. It is likely that LPF is a T-cell mitogen, but LPF, unlike PHA, does not cause maximal stimulation of isolated T-cell enriched populations unless accessory cells are present.

Addendum. While this manuscript was in revision, Andersen et al. reported that *B. pertussis* cells and partially purified LPF stimulated normal human PBL and suggested that the responding cells were T cells (22).

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REFERENCES

1. Lagergren, J. 1963. The white blood cell count and the erythrocyte sedimentation rate in pertussis. *Acta Paediatr. Scand.* **52:** 405–409.
2. Morse, S. I., and K. K. Bray. 1969. The occurrence and properties of leukocytosis and lymphocytosis-stimulating material in the supernatant fluids of *Bordetella pertussis* cultures. *J. Exp. Med.* **129:** 523–558.
3. Morse, S. I., and J. H. Morse. 1976. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. *J. Exp. Med.* **143:** 1483–1502.
4. Kong, A. S., and S. I. Morse. 1975. The mitogenic response of mouse lymphocytes to the lymphocytosis-promoting factor (LPF) of *Bordetella pertussis*. *Fed. Proc.* **34:** 951.
5. Kong, A. S., and S. I. Morse. 1977. The *in vitro* effects of *B. pertussis* lymphocytosis-promoting factor (LPF) on murine lymphocytes. I. Proliferative response. *J. Exp. Med.* **145:** 151–162.
6. Kong, A. S., and S. I. Morse. 1977. The *in vitro* effects of *B. pertussis* lymphocytosis-promoting factor (LPF) on murine lymphocytes. II. Nature of the responding cells. *J. Exp. Med.* **145:** 163–174.
7. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97): 77–89.
8. Harrison, M. R., G. B. Thurman, and G. M. Thomas. 1974. A simple and versatile harvesting device for processing radioactive label incorporated into and/or released from cells in microculture. *J. Immunol. Methods.* **4:** 11–16.
9. Salem, N. B., and J. H. Morse. 1976. Lymphocyte response to mitogens in progressive systemic sclerosis. *Arthritis Rheum.* **19:** 875–882.
10. Wybran, J., M. C. Carr, and H. H. Fudenberg. 1972. The human rosette-forming cell as a marker of a population of thymus-derived cells. *J. Clin. Invest.* **51:** 2537–2543.
11. Bianco, C., R. Patrik, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterization. *J. Exp. Med.* **132:** 702–720.
12. Gupta, S., and M. H. Grieco. 1975. Rosette formation with mouse erythrocytes: probable marker for human B lymphocytes. *Int. Arch. Allergy Appl. Immunol.* **49:** 734–742.
13. Zacharski, L. R., and J. W. Linman. 1969. Chronic lymphocytic leukemia versus chronic lymphosarcoma cell leukemia: analysis of 496 cases. *Am. J. Med.* **47:** 75–81.
14. Carbone, P. P., H. S. Kaplan, K. Musshoff, D. W. Smithers, and M. Tubiana. 1971. Report of the committee on Hodgkin's disease staging classification. *Cancer Res.* **31:** 1860–1861.
15. Rai, K. R., A. Sawitsky, E. P. Cronkhite, A. D. Chanana, R. N. Levy, and B. S. Pasternack. 1975. Clinical staging of chronic lymphocytic leukemia. *Blood.* **46:** 219–234.
16. Brouet, J-C., G. Flandrin, and M. Seligmann. 1973. Indications of the thymus-derived nature of the proliferating cells in six patients with Sezary's syndrome. *N. Engl. J. Med.* **289:** 341–344.
17. Lambert, H. J. 1965. Epidemiology of a small pertussis outbreak in Kent County, Michigan. *Public Health Rep.* **80:** 365–369.
18. Morse, S. I., and S. K. Riester. 1967. Studies on the leukocytosis and lymphocytosis induced by *Bordetella pertussis*. II. The effect of pertussis vaccine on the thoracic duct lymph and lymphocytes of mice. *J. Exp. Med.* **125:** 619–628.
19. Shohat, B., and H. Joshua. 1976. Formation of mouse red cell rosettes by lymphocytes from patients with chronic lymphatic leukemia and assessment of T cell function. *Clin. Immunol. Immunopathol.* **6:** 389–393.
20. Broder, S., R. L. Edelson, M. A. Lutzner, D. L. Nelson, R. P. MacDermott, M. E. Durm, C. K. Goldman, B. D. Meade, and T. A. Waldmann. 1976. The Sezary syndrome: a malignant proliferation of helper T cells. *J. Clin. Invest.* **58:** 1297–1306.
21. Ho, M-K., A. S. Kong, and S. I. Morse. 1977. Influence of non-responding cells on the mitogenic response of murine T cells to the lymphocytosis-promoting factor of *Bordetella pertussis*. *Fed. Proc.* **36:** 1323.
22. Andersen, V., J. B. Hertz, S. F. Sprensen, P. Backgaard, P. E. Christensen, W. Ramhj, G. A. Hansen, A. E. Wardlaw, and N. Y. Sato. 1970. *In vitro* stimulation of human lymphocytes by *Bordetella pertussis*. *Acta Pathol. Microbiol. Scand. Sect. C. Immunol.* **85:** 65–72.