THE IN VITRO EFFECTS OF *BORDETELLA PERTUSSIS* LYMPHOCYTOSIS-PROMOTING FACTOR ON MURINE LYMPHOCYTES

I. Proliferative Response*

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The marked increase in the number of circulating lymphocytes induced in experimental animals by the lymphocytosis-promoting factor (LPF)[1] of phase I *Bordetella pertussis* is due to redistribution of cells from lymphoid tissues into the blood (1–4). The redistribution involves in large part the failure of recirculating lymphocytes to "home" into lymph nodes at a normal rate (5, 6). However, the mechanism responsible for failure of normal homing is unknown. In addition, although LPF binds to a variety of cells, including lymphocytes (7), little is known of the consequences of this interaction. Parker and Morse (8) did find, however, that fractions of culture supernates of *B. pertussis* rich in LPF inhibited the accumulation of cyclic adenosine 3′,5′-monophosphate (cAMP) in human lymphocytes which is normally induced by isoproterenol and prostaglandin E1.

Recently, procedures have been developed in this laboratory for the isolation of LPF (9) which was found to be a protein which not only causes lymphocytosis but also sensitizes mice to histamine and, in addition, produces a markedly diminished hyperglycemic response to epinephrine.

The availability of highly purified preparations has made it possible to pursue studies on the in vitro effects of LPF. In the studies reported herein, the interaction between LPF and cultured murine lymphocytes was examined. Surprisingly, LPF proved to be a potent lymphocyte mitogen in vitro, despite the fact that pertussis-induced lymphocytosis is not accompanied by lymphocyte proliferation (1). A preliminary account of these findings has been presented previously (10).

Materials and Methods

Animals. Inbred female CBA/J (CBA) mice, 8-to-12 wk of age, were employed for most of the experiments and were obtained from The Jackson Laboratory, Bar Harbor, Maine, or from

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[1] *Abbreviations used in this paper*: Con A, concanavalin A; LPF, lymphocytosis-promoting factor; LPS, lipopolysaccharide; PHA, phytohemagglutinin.

Jackson stock bred in this department. C57BL/6J (C57), BALB/cJ (BALB), C3H/HeJ (C3H) inbred animals were also obtained from The Jackson Laboratory, while outbred Albany mice were from the Griffin Laboratory, Albany, N. Y. Conventional and germ-free CD-1 animals were purchased from Charles River Mouse Farm Inc., Wilmington, Mass.

LPF. LPF was isolated from culture supernatant fluids of phase I *B. pertussis* strain NIH 114 by previously described methods (2). Intravenous injection of 0.5 μg of LPF protein into CD-1 mice resulted in a leukocytosis of 110,000–160,000 cells/mm^3 3 days later, and small lymphocytes comprised 60–70% of the population.

Mitogens. Phytohemagglutinin-M (PHA-M) was obtained from Difco Laboratories, Detroit, Mich., and after reconstitution was stored in aliquots at −20°C. PHA-P (Difco Laboratories) was used in some experiments with qualitatively identical results. Con A was obtained from Calbiochem, San Diego, Calif. LPS, isolated from *Salmonella typhosa* by the phenol-water technique (11), was kindly supplied by Dr. B. M. Sultzer of this Department.

Cell Cultures. The medium used throughout, unless other specified, was RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Reheis Chemical Co., Phoenix, Ariz.), penicillin (100 U/ml), and streptomycin (100 μg/ml). Mice were killed by cervical dislocation, and isolated cells were prepared from spleen, inguinal, and axillary lymph nodes, and thymus by teasing the organs with forceps into medium. Tissue fragments were allowed to sediment spontaneously, and the supernate containing single cells was aspirated.

In some experiments suspensions of bone marrow cells were employed. These were prepared from femora that were freed from adhering muscle and fascia. The ends of the bones were cut with scissors and the marrow plug expelled with a 26-gauge needle. The cells were dispersed with a Pasteur pipette.

Cell suspensions were centrifuged, washed once, and resuspended in fresh medium. Viability was assessed with the trypan blue dye exclusion test, and only preparations with greater than 90% cell viability were utilized.

Cell Cultures. In most experiments the cell suspensions were adjusted to 4×10^6 viable cells/ml and mixed with an equal volume of medium containing mitogen, or the solvent used for the mitogen. Then 0.25 ml of the mixture, containing 0.5×10^6 cells, was added to wells of flat bottom microtiter plates (Model IS FB-96-TC, Linbro Chemical Co., New Haven, Conn). Triplicate cultures were incubated at 37°C in a humid atmosphere of 5% CO_2 in air for the indicated time periods.

Determination of DNA Synthesis. 8 h before harvest, 2.0 μCi of [^{3}H]thymidine, sp act 6.0 Ci/mM (Schwarz/Mann, Orangeburg, N. Y.), was added to the cultures in a vol of 0.02 ml. The cultures were harvested on glass fiber filter papers (grade 934 AH, Reeve Angel, Clifton, N. J.) with an automated sampler (Otto Hiller Co., Madison, Wisc.) and the filters washed with saline. The dried filters were placed in 5 ml of an Omnifluor-toluene scintillation mixture (New England Nuclear, Boston, Mass.) and counted. The responses are presented as mean cpm ± 1 SD of triplicate samples less background and occasionally as the stimulation index (SI) where SI = (mean cpm of stimulated cultures)/(mean cpm of control cultures).

Light Microscopy. Morphological examination of cultured cells for evidence of transformation was performed on Wright-Giemsa stained preparations which had been sedimented with a Shandon Cytocentrifuge (Rainen Instruments Co., Fort Lee, N. J.).

Results

Effect of Various Concentrations of LPF on the Proliferation of CBA Lymph Node and Spleen Cells. Various quantities of LPF were added to cultures containing 0.5×10^6 CBA lymph node or spleen cells in a final vol of 0.25 ml, and the incorporation of [^{3}H]thymidine at 72 h was determined. This time period was selected on the basis of results obtained in preliminary experiments. As can be seen in Fig. 1, there was a relatively sharp optimum dose of 0.5 μg of LPF per culture for lymph node cells, while the optimum dose for spleen cells was 0.25–0.5 μg. However, the overall response of lymph node cells was slightly less dose dependent, good stimulation being seen at doses of 0.25–0.75 μg. At higher

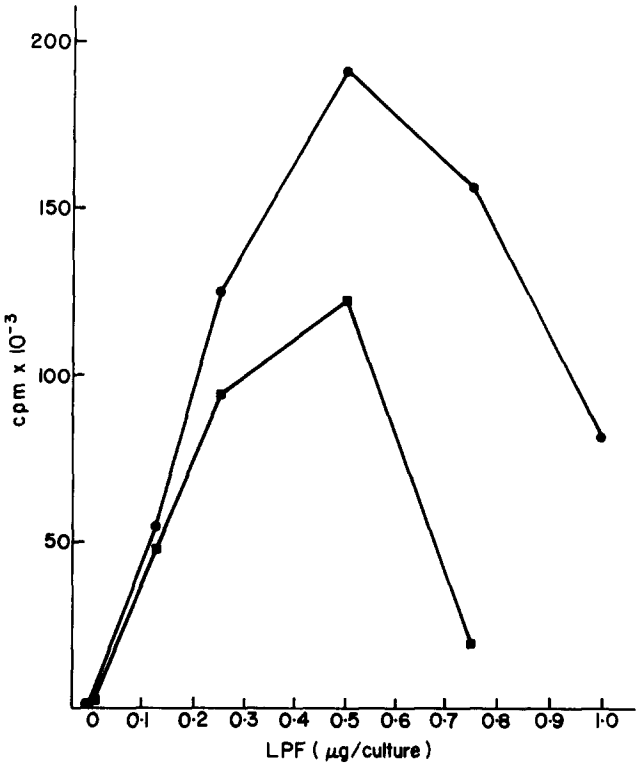

FIG. 1. The incorporation of [^{3}H]thymidine by CBA lymph node cells (●—●) and spleen cells (■—■) in response to various quantities of LPF. Cultures contained 0.5 × 10^6 cells in 0.25 ml, and results are shown as the mean cpm × 10^{-3} minus background after 72 h of incubation.

concentrations of LPF there was marked suppression of the proliferative response (Fig. 1), but viability studies indicated that the suppression was not a manifestation of cytotoxicity of LPF.

Time-Course of the Proliferative Response to LPF. As shown in Fig. 2, when CBA spleen cells were incubated with optimum doses of 0.25 or 0.5 μg of LPF per culture, virtually no proliferative response could be detected at 24 h. Peak incorporation of [^{3}H]thymidine was found at 72 h, while at 96 h incorporation was markedly diminished.

Morphologic findings paralleled the results of estimation of proliferation by [^{3}H]thymidine incorporation. After 72 h of incubation of spleen cells with 0.5 μg of LPF, 70–90% of the cells were blast forms and mitotic figures were frequent (Fig. 3).

In the case of lymph node cells (Fig. 2), the response to the optimum dose of 0.5 μg of LPF was maximal at 72 h, whereas for the higher dose of 0.75 μg the peak was usually at 48 h. In the presence of 0.25 μg of LPF the response increased successively over 96 h although not in a strictly linear fashion.

Comparison of the Mitogenic Effects of LPF, PHA, and Concanavalin A (Con A) on CBA Spleen Cells. The comparative ability of optimum amounts of LPF, PHA, and Con A to stimulate 0.5 × 10^6 CBA spleen cells was tested at 48 and 72

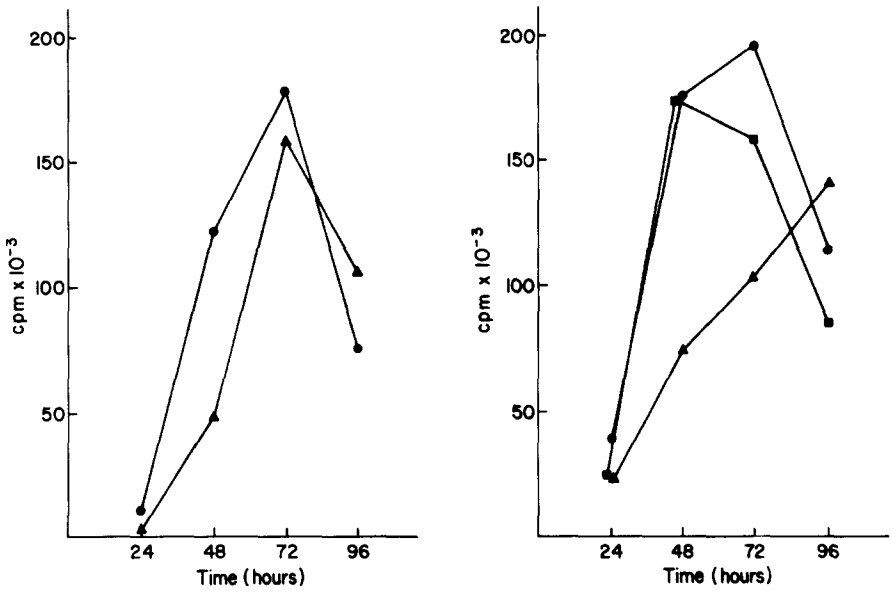

FIG. 2. [^{3}H]thymidine incorporation at various time periods by CBA spleen cells in response to 0.25 μg (▲—▲) and 0.5 μg (●—●) of LPF per culture (left), and by CBA lymph node cells to 0.25, 0.5, and 0.75 μg (■—■) of LPF (right). Cultures contained 0.5 × 10^6 cells in 0.25 ml. Results are expressed as mean cpm × 10^{-3} minus background.

h of culture. Under these conditions it was found that LPF was much more potent than PHA, but somewhat less than Con A (Table I).

Effect of Cell Concentration on the Response to LPF. It is well known that the concentration of lymphocytes markedly affects the extent of proliferation in mixed lymphocyte cultures (12) as well as reactivity to the phytomitogen PHA (13). Therefore, the response of differing numbers of CBA spleen cells in 0.25 ml to 0.5 μg of LPF, a dose optimum for 0.5 × 10^6 cells, was examined. As can be seen in Table II, 0.25 × 10^6 cells showed little response to this amount of LPF, whereas there was marked response at higher cell concentrations without significant differences in the extent of [^{3}H]thymidine incorporation. This result stood in marked contrast to the decreased reactivity of the highest cell concentration, 2.0 × 10^6/0.25 ml, to PHA (Table II).

Evidence that LPF is a Nonspecific Mitogen. A critical question was whether the response to LPF represents an in vitro manifestation of antigen stimulation of cells presensitized either to LPF or to antigens cross-reactive with it, or whether LPF is a nonspecific, polyclonal mitogen. Two types of experiments suggested that presensitized cells were not responsible for the reaction.

Firstly, as shown in Table III, spleen cells from a variety of mouse strains were reactive. Secondly, with respect to both LPF and PHA, spleen cells from individual axenic CD-1 mice showed the same range of reactivity as did spleen cells from conventionally reared CD-animals (Table IV).

Effect of LPF on Thymocytes from Normal and Cortisone-Treated Mice. For these experiments BALB mice were employed since their thymocytes tend to be more reactive to mitogens than those of other strains. Yet as shown in Fig. 4, 0.5 × 10^6 BALB thymocytes did not respond to a wide range of LPF concentrations,

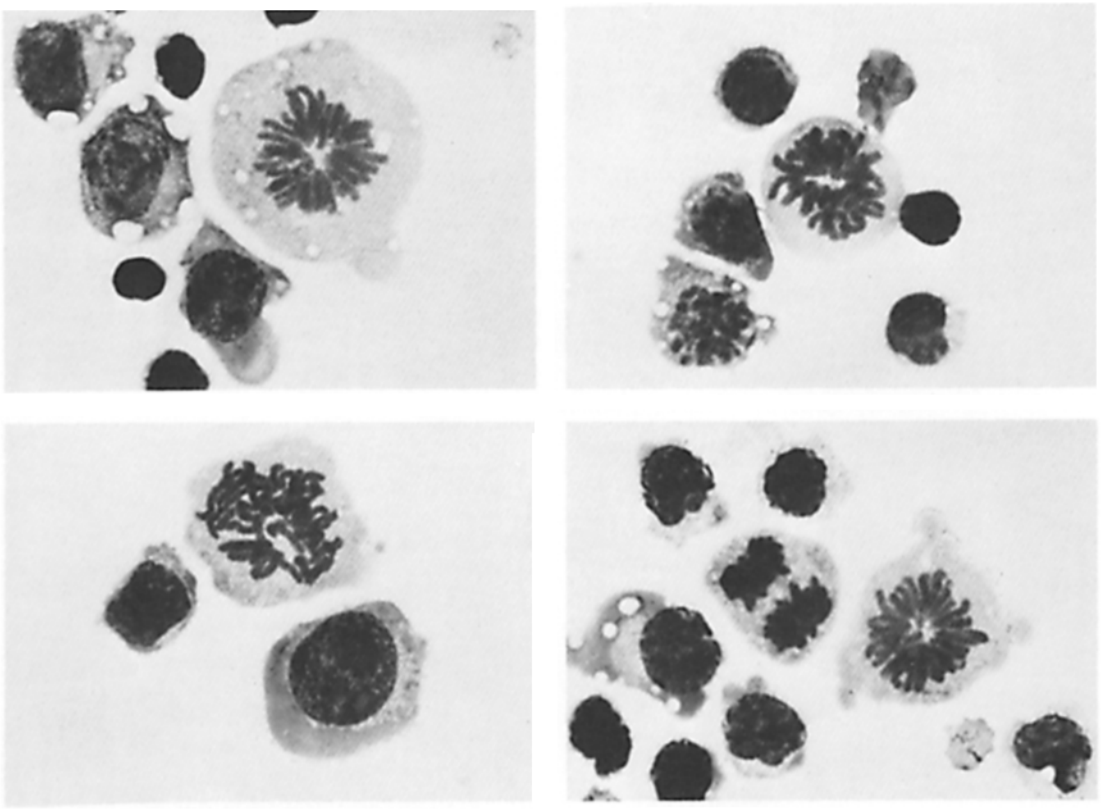

FIG. 3. Morphology of CBA spleen cells, 0.5 × 10^6 cells/0.25 ml, after 72 h of incubation with 0.5 μg of LPF. Wright-Giemsa × 1250.

TABLE I

[^{3}H]thymidine Incorporation by CBA Spleen Cells in Response to Mitogens

Mitogen	Dose	[^{3}H]thymidine incorporation	
		48 h	72 h
LPF	0.5 μg	88,926 ± 4,718*	150,069 ± 10,655
PHA	1.25 μl	31,356 ± 1,147	62,713 ± 2,843
	2.5 μl	37,949 ± 3,726	52,267 ± 2,228
	3.75 μl	33,318 ± 4,774	57,894 ± 3,434
Con A	0.25 μg	112,994 ± 4,398	170,238 ± 2,446
	0.5 μg	138,309 ± 2,570	180,357 ± 6,077
	0.75 μg	120,033 ± 3,619	172,438 ± 2,960
None	–	2,244 ± 71	1,597 ± 180

* Mean cpm ± SD of triplicate cultures.

whereas they did respond to Con A. However, when the concentration of thymocytes was increased to 1.0–2.0 × 10^6 cells/0.25 ml, slight but definite proliferation occurred (Table V). Since it has been shown that while the vast majority of thymocytes do not respond to PHA, there exists a population of

TABLE II

[^{3}H]thymidine Incorporation by Varying Numbers of CBA Spleen Cells in Response to LPF and PHA

Mitogen	Number of cells per culture			
	0.25×10^6	0.5×10^6	1.0×10^6	2.0×10^6
None	933 ± 112*	2,252 ± 394	7,901 ± 1,260	18,176 ± 1,771
LPF (0.5 μg)	24,751 ± 1,553	173,389 ± 4,731	198,715 ± 2,014	171,778 ± 7,016
PHA (2.5 μl)	26,857 ± 6,806	81,302 ± 2,300	115,564 ± 14,152	42,258 ± 12,695

* Mean cpm ± SD of triplicate cultures at 72 h.

TABLE III

[^{3}H]thymidine Incorporation by Spleen Cells from Different Mouse Strains in Response to LPF

Mouse strain	Medium alone	LPF (0.5 μg)	SI*
CBA/J	1,597 ± 180‡	150,069 ± 10,665	93.9
C57BL/6J	471 ± 124	119,601 ± 2,424	253.9
BALB/cJ	1,461 ± 477	92,257 ± 4,348	63.1
DBA/2J	2,600 ± 646	78,469 ± 2,328	30.1
C3H/HeJ	986 ± 214	78,879 ± 8,344	79.9
CD-1	2,221 ± 706	150,840 ± 15,248	67.9
Albany	1,965 ± 375	101,090 ± 7,009	51.4

* Stimulation index.

‡ Mean cpm ± SD of triplicate cultures at 72 h.

TABLE IV

[^{3}H]thymidine Incorporation by Spleen Cells from Individual Normal and Axenic CD-1 Mice in Response to LPF and PHA

	Mitogen added			
	None	LPF (0.25 μg)	LPF (0.5 μg)	PHA (2.5 μl)
Axenic CD-1	528 ± 109*	11,817 ± 727	53,053 ± 11,463	58,055 ± 13,044
	2,458 ± 820	107,572 ± 18,642	157,943 ± 11,342	69,468 ± 8,479
	1,566 ± 110	78,110 ± 12,991	108,404 ± 12,318	53,145 ± 8,128
	2,200 ± 132	39,130 ± 12,247	114,964 ± 1,791	57,558 ± 5,377
	2,059 ± 864	53,391 ± 10,811	132,171 ± 8,432	58,193 ± 31,955
Normal CD-1	2,221 ± 706	56,198 ± 11,348	150,840 ± 15,248	120,718 ± 9,846
	454 ± 60	11,418 ± 2,316	40,838 ± 14,783	22,112 ± 3,286
	1,349 ± 207	54,934 ± 15,474	171,406 ± 6,561	80,650 ± 4,606

* Mean cpm ± 1 SD of triplicate cultures harvested at 72 h.

cortisone-resistant thymocytes which are stimulated by this mitogen (14), it was important to determine whether the same was true with respect to LPF.

It can be seen in Table VI that, as in the case of PHA, 30 h after injection of 5 mg of cortisone acetate there was a residual population of steroid-resistant cells which responded to LPF.

Effect of LPF on Bone Marrow Cells. Fig. 5 illustrates that whereas CBA bone marrow cells incorporated [^{3}H]thymidine when incubated for 72 h with LPS, there was no response to several concentrations of LPF.

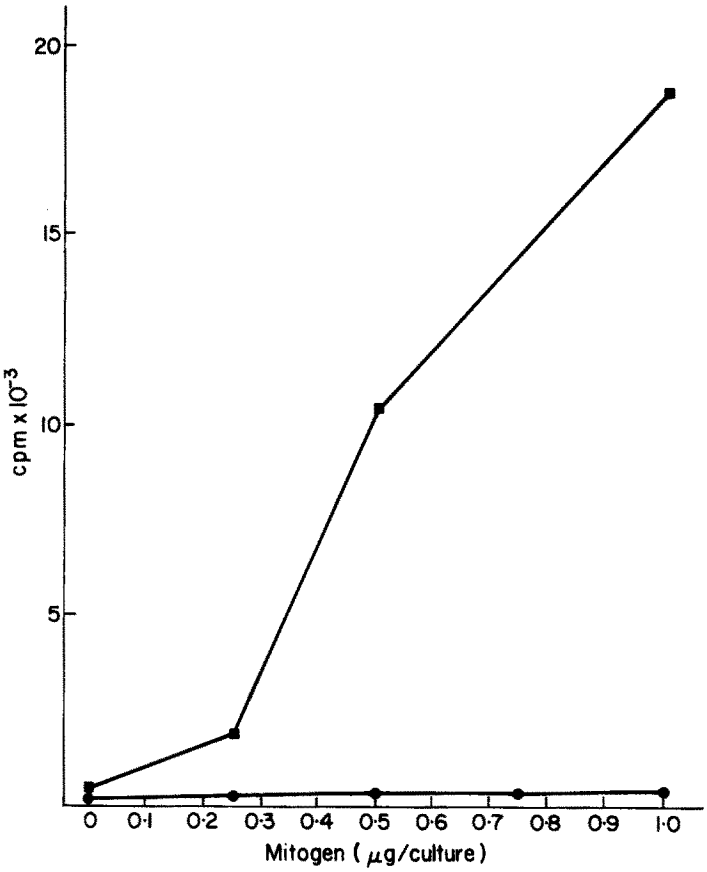

FIG. 4. [^{3}H]thymidine incorporation by BALB thymocytes 0.5 × 10^6 cells/culture after 72 h of incubation with Con A (■–■) or LPF (●–●).

TABLE V

[^{3}H]thymidine Incorporation by Varying Numbers of BALB Thymus Cells in Response to LPF and Con A

Mitogen	Number of cells per culture			
	0.25 × 10^6	0.5 × 10^6	1.0 × 10^6	2.0 + 10^6
None	43 ± 5*	76 ± 11	117 ± 5	150 ± 14
LPF (0.5 μg)	169 ± 6	437 ± 53	1,324 ± 134	3,948 ± 811
Con A (0.5 μg)	3,710 ± 270	28,000 ± 4,135	114,949 ± 17,194	183,242 ± 26,554

* Mean cpm ± SD of triplicate cultures at 48 h.

Abrogation of the Proliferative Response by LPF Antiserum. Monospecific LPF antiserum was prepared in rabbits as described previously (9). When added at the initiation of the in vitro cultures, it was found that as little as 150 nl of antiserum specifically nullified the proliferative response to LPF, whereas normal rabbit serum had no effect. Moreover, the LPF antiserum affected neither background counts nor PHA reactivity.

This reagent proved useful in assessing the duration of contact between cells

TABLE VI
Mitogen Response of Thymocytes from Normal and Cortisone Acetate-Treated BALB Mice

Treatment	Mitogen				
	LPF (0.5 μg)	LPF (0.75 μg)	PHA (2.5 μl)	Con A (1 μg)	None
None	261 ± 48*	360 ± 50	497 ± 20	18,700 ± 7,577	65 ± 12
Cortisone	25,826 ± 510	37,203 ± 3,569	35,337 ± 4,229	198,795 ± 8,081	933 ± 19

* Mean cpm ± SD at 48 h.

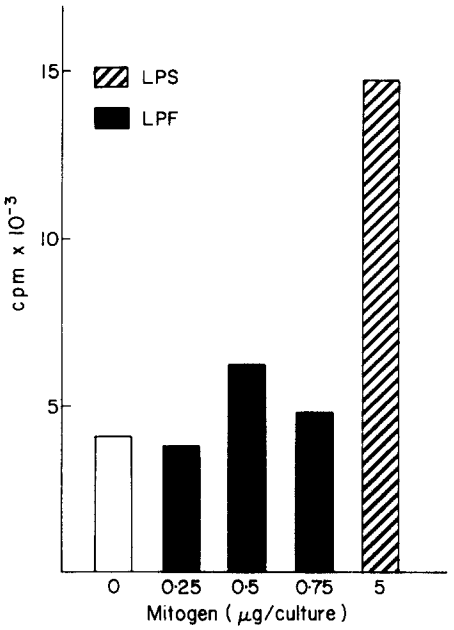

FIG. 5. [^{3}H]thymidine incorporation by 0.5 × 10^6 CBA bone marrow cells after incubation for 72 h with either LPF or LPS.

and LPF which was required to initiate a proliferative reaction. Antiserum (625 nl) was added at various times, and the incorporation of [^{3}H]thymidine by CBA spleen cells at 72 h was compared to that in cultures containing 625 nl of normal rabbit serum.

As seen in Fig. 6, complete abrogation of the response occurred when anti-LPF was added up to 3 h after cultures were initiated. There was successively less inhibition when antiserum was added at 12 and at 24 h, but even at 48 h the introduction of antiserum resulted in a 40% reduction in [^{3}H]thymidine incorporation at 72 h. Normal rabbit serum had no inhibiting effect, and LPF antiserum did not affect the response to PHA.

These results were supported by other experiments in which the cells were washed free of LPF at various time periods and resuspended in fresh medium. Although the data are not strictly comparable owing to differences in design and the difficulty in washing out cell-bound LPF, it was found that removal of extracellular LPF as late as 24 h resulted in a decreased response at 72 h.

The anti-LPF serum also proved useful in assessing whether LPF induced the

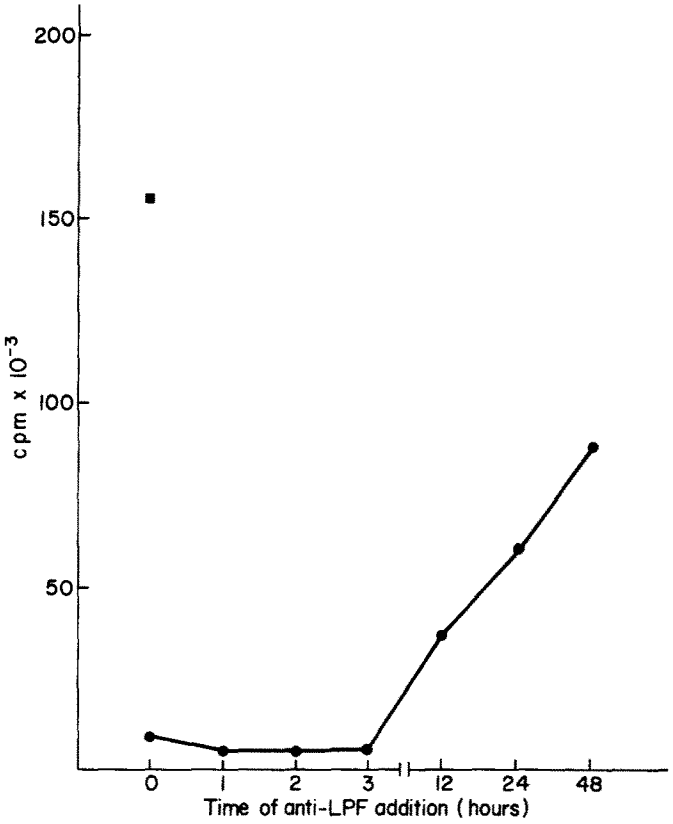

FIG. 6. The effect of the addition of 625 nl of rabbit LPF antiserum (●–●) at various time periods on the incorporation of [^{3}H]thymidine by cultures of CBA spleen cells containing 0.5 μg of LPF. In control cultures normal rabbit serum (■) was added at the initiation of the cultures. The data are expressed as mean cpm × 10^{-3}, minus background, after a total of 72 h in culture.

production of a mitogenic factor from stimulated lymphocytes. CBA spleen cells were incubated with LPF for various time periods. The cells were then removed by centrifugation, and the supernatant fluids, to which LPF antiserum had been added, were used in fresh spleen cell cultures. No mitogenic effect was found.

Discussion

Previous studies have clearly shown that the lymphocytosis induced by LPF in experimental animals is due to redistribution of cells and is not the result of lymphocyte proliferation (1–6). Yet, in the in vitro studies reported herein, LPF was found to be a potent mitogen for cultured murine lymphocytes.

It is unlikely that the mitogenic principle of phase I *B. pertussis* is a contaminant present in LPF preparation. In unpublished experiments, not only was the total LPF fraction mitogenic, but so was the eluate from the major band found when undissociated LPF is subjected to polyacrylamide electrophoresis. The same eluate, as previously shown (9), induced lymphocytosis, histamine sensitization, and epinephrine unresponsiveness in mice. Further confirmation that an

identical substance causes the in vivo and in vitro effects is derived from the finding that a monospecific LPF antiserum abrogated the diverse reactions.

The most likely explanation for the discrepancy between the in vivo and in vitro effects lies in the different dose of LPF required for in vitro stimulation compared with that which induces lymphocytosis. Optimum lymphocytosis in mice is caused by the injection of 0.5–1.0 μg of LPF, whereas there is a sharp optimum of approximately 0.5 μg required for the in vitro proliferation of 0.5×10^6 lymphocytes; markedly diminished responses occur when less than 0.25 μg is used. Thus, the dilution of LPF in the plasma volume alone accounts for the lack of DNA replication in the lymphocytes of intact mice. Moreover, while there is no information on the distribution of LPF in cellular and extracellular compartments, it is known that LPF reversibly attaches to a variety of mammalian cells (7) which would further reduce the effective concentration available to the lymphocytes. Although it is possible that exceedingly large doses of LPF might produce lymphocyte proliferation in vivo, it is obvious that proliferation is not mandatory for lymphocytosis. In this regard it is of interest that while LPF induces lymphocytosis in nude mice (9), LPF does not cause in vitro stimulation of either spleen cells or lymph node cells from these animals (15).

Although there is no known animal reservoir of *B. pertussis*, it was possible that sensitization of lymphocytes to bacterial antigens cross-reacting with LPF could have occurred, and that the mitogenic response to LPF was due to specific antigen stimulation. This possibility was rendered unlikely by the finding that spleen cells from germ-free mice responded as well to LPF as did spleen cells from conventionally reared mice of the same strain. A variety of bacteriological procedures were employed to ensure that the axenic mice were indeed free of culturable microorganisms. It might still be argued that the mice were sensitized to cross-reactive viral or food antigens. This idea seems untenable for several reasons. Spleen cells from a number of mouse strains obtained from several sources responded to approximately the same extent. In addition, serological tests did not reveal evidence of exposure to LPF or cross-reactive antigens. The magnitude of the proliferative response, which exceeds that produced by PHA and is only slightly less than that seen with Con A, is also far greater than that generally observed with antigen-sensitized mouse lymphoid cells. The results of experiments performed with human lymphocytes also support the thesis that LPF is a nonspecific mitogen (J. H. Morse, A. S. Kong, J. Lindenbaum, and S. I. Morse, unpublished observations).

The availability of specific LPF antiserum, which blocks the mitogenic response, permitted analysis of the time-dependent requirement for serologically active LPF in the lymphocyte cultures. A minimum period of several hours of exposure to LPF, in the absence of antiserum, was required for any response to occur. It was striking that when LPF antiserum, but not normal rabbit serum, was added as late as 48 h, subsequent diminution of [^{3}H]thymidine incorporation occurred. The data do not allow one to determine whether this was due to neutralization of extracellular LPF or to the consequences of an interaction between the antiserum and LPF which might be cell-bound.

B. pertussis is a gram-negative organism which contains lipopolysaccharide endotoxin (LPS), but a number of experiments indicated that the observed response of murine lymphocytes was not due to contamination of LPF by LPS.

Chemical analysis of LPF reveals no lipid (9) and, furthermore, LPF did not cause cultured bone marrow cells to incorporate [^{3}H]thymidine, whereas LPS from *S. typhosa* did. LPS prepared from the homologous strain of *B. pertussis* by the phenol-water technique (11), unlike LPF, also stimulated bone marrow cells (unpublished observations).

Spleen cells and lymph node cells were responsive to LPF, but normal thymocytes were not stimulated by LPF except at high cell concentrations. However, there existed a cortisone-resistant population of thymocytes which was responsive to LPF. In contrast to LPF, Con A-stimulated normal thymocytes, whereas PHA, like LPF, stimulated only cortisone-resistant thymus cells.

In an accompanying paper it will be shown that the lymphocytes stimulated by LPF are T cells sensitive to Thy-1 antiserum, which comprise a population different from those that react to PHA. Moreover, adherent, Thy-1 insensitive cells were found to be required for initiation of the response.

Summary

The lymphocytosis-promoting factor of *Bordetella pertussis* is a potent mitogen for murine lymphocytes in vitro. The stimulatory response was not the result of specific antigen stimulation. Spleen and lymph node cells were responsive, whereas normal thymocytes were unresponsive. However, DNA replication was induced in cortisone-resistant thymocytes by lymphocytosis-promoting factor (LPF). Bone marrow cells were not stimulated by LPF.

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