STUDIES ON A NEW LYMPHOCYTE MITOGEN FROM *BORDETELLA PERTUSSIS*

I. Induction of Proliferation and Polyclonal Antibody Formation*

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The major interest of our laboratory in recent years has been the isolation of components of *Bordetella pertussis* and the study of their effects. One of these components, lymphocytosis-promoting factor (LPF),[1] has been purified to homogeneity (1). LPF is responsible for the lymphocytosis observed in a number of animals after injection of phase I *Bordetella pertussis*. Moreover, it induces hypoglycemia and sensitizes mice to the lethal effects of histamine. In vitro, LPF is a potent mitogen for both mouse and human lymphocytes (2, 3). It is known, at least in mice, that LPF is a B-dependent T mitogen. Hence, all the proliferating cells in LPF cultures are T lymphocytes, but T cells can only respond to LPF in the presence of helper B lymphocytes (4). In vitro culture of lymphocytes with LPF also results in the production of cytotoxic effector lymphocytes with specificity for allogeneic and syngeneic tumor cells as well as allogeneic normal cells (5).

The last step in the purification of LPF involved gel filtration on Sephadex G-150 columns. Three protein peaks were detected after elution. LPF was recovered in the first included peak, whereas the excluded peak contained the hemagglutinin (HA) but no LPF activity. We now report that, in addition to HA, a potent mitogen distinct from LPF is also present in this fraction excluded from G-150. In contrast with LPF, this mitogen induces the proliferation of mouse B lymphocytes, but not T cells. Hence this substance is termed pertussis B mitogen (PBM). PBM is also mitogenic for human peripheral blood lymphocytes and cord blood lymphocytes. Moreover, it is a polyclonal activator for both mouse and human lymphocytes in vitro.

Materials and Methods

Mice. CBA/J, BALB/c, C3H/HeJ, and DBA/2J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). CBA/N mice were bred in this department from National

The authors wish to dedicate this paper to the memory of Dr. Stephen I. Morse, our mentor, for his invaluable contribution to the field of Microbiology and for being a constant source of inspiration and support throughout the ten years he served as chairman and professor of our department.

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; Con A, concanavalin A; EP, endotoxin protein; FCS, fetal calf serum; HA, hemagglutinin of *Bordetella pertussis*; HPBL, human peripheral blood lymphocyte; LPF, lymphocytosis-promoting factor; LPS, lipopolysaccharide; 2-ME, β-mercaptoethanol; NWSM, *Nocardia* water-soluble mitogen; PAGE, polyacrylamide gel electrophoresis; PBM, pertussis B mitogen; PFC, plaque-forming cell; PHA-P, phytohemagglutinin-P; PWM, pokeweed mitogen; SDS, sodium dodecyl sulfate; sIg, surface immunoglobulin; SRBC, sheep red blood cell; TNP, trinitrophenyl; TxB, thymectomized, B cell reconstituted.

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Institutes of Health stock, whereas conventional and germfree CD-1 animals were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Unless otherwise stated, female CBA/J mice, 8–14 wk of age, were employed for all experiments. TxB mice were adult thymectomized, irradiated, and reconstituted with 14- to 16-d fetal liver cells as previously described (6).

Mouse Lymphocyte Cultures. Lymphocytes from spleen, peripheral lymph node, bone marrow, and thymus were prepared as described (4) and cultured in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Microbiological Associates, Walkersville, Md.), 100 U/ml penicillin, and 100 μg/ml streptomycin unless specified.

For determination of DNA synthesis, 5×10^5 lymphocytes in 0.25 ml of medium were cultured in microtiter plates (IS-FB 96; Linbro Chemical Co., Harden, Conn.), pulsed with [^{3}H]thymidine, harvested, and counted as described (4). The [^{3}H]thymidine incorporation was expressed as the mean counts per minute $\times 10^{-3} \pm$ SD of triplicate samples.

For assay of antibody-forming cells, a modified method of Nilsson et al. was adopted (7). Briefly, triplicate cultures containing 10×10^6 lymphocytes in 1 ml of RPMI-5% FCS were incubated with or without mitogens in 10- × 35-mm plastic petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 37°C. The dishes were placed on a rocking platform (8 oscillations/min) in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2. After 48 h of incubation, cells from triplicate cultures were scraped off the dishes, pooled, and washed three times in medium. Antibody-forming cells were enumerated by a modified hemolytic plaque assay (7) using sheep erythrocytes and trinitrophenyl (TNP)-conjugated sheep erythrocytes (8).

Human Lymphocyte Cultures. Heparinized venous blood was obtained from normal healthy donors. Blood mononuclear cells were collected by a modification of Boyum's method (9). Briefly, the blood was mixed with an equal volume of sterile saline. 2 vol of this blood-saline solution was layered on 1 vol of Ficoll-Conray solution prepared by mixing 1 vol of 33.4% Conray (meglumine iothalamate; Mallinckrodt Inc., St. Louis, Mo.) to 2.4 vol of 9% Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.). After centrifuging for 30 min at 4°C, cells were collected from the interface and washed three times with serum-free RPMI-1640. When assayed for proliferative responses, triplicate cultures of 1.25×10^5 cells in 0.25 ml of RPMI-1640-5 % FCS were dispensed in Linbro microtiter plates (IS-FB 96) and incubated in 5% CO_2, 95% air at 37°C for 120 h. The cells were pulsed and processed as for mouse cells. Cord blood was processed in a similar fashion.

For polyclonal activation, the procedure of Fauci and Pratt (10) was employed. Human peripheral blood lymphocytes (HPBL) were resuspended to 2×10^6/ml in RPMI-1640 supplemented with 10% fresh pooled AB serum obtained from healthy donors. Triplicate cultures of 2×10^6 HPBL were incubated with the appropriate mitogens in 12- × 75-mm plastic tubes at 37°C in a humid atmosphere of 5% CO_2, 95% air. At 120 h cells from triplicate cultures were pooled and washed three times with phosphate-buffered saline, pH 7.2. Antibody-forming cells against sheep erythrocytes or TNP-conjugated sheep erythrocytes were assayed as described for mouse cells.

Mitogens. LPF was purified from the culture supernatant fluids of phase I *Bordetella pertussis,* strain 114, as described (1). Briefly, solid $(NH_4)_2SO_4$ was added to culture supernates to 90% saturation at 4°C and pH 6.4. Then the $(NH_4)_2SO_4$ precipitates were collected and dialyzed against water. The water-insoluble material was resuspended in 0.1 M Tris-0.5M NaCl, pH 10. Any insoluble material was discarded. The soluble fraction was loaded on a CsCl gradient of densities 1.5, 1.3, 1.25, and 1.2 and after centrifuging at 50,000 *g* for 3.5 h, the load volume was removed and passed through Sephadex G-150. Three protein peaks were eluted. LPF was isolated and purified from the first included peak, whereas PBM and HA were found in the excluded peak. Optimal concentrations of mitogens per culture of mouse lymphocytes are as follows: LPF, 0.5 μg; PBM, 0.5 μg; phytohemagglutinin-P (PHA-P) (Difco Laboratories, (Detroit, Mich.), 0.18 μl; concanavalin A (Con A) (Pharmacia Fine Chemicals), 0.5 μg. In early experiments, lipopolysaccharide (LPS) prepared from *B. pertussis* by the method Nakase et al. (11) was used at 5 μg/culture whereas later experiments employed *S. typhosa* LPS (phenol-water preparation; Difco Laboratories) at 1.25 μg/culture.

For mitogenic stimulation of HPBL, the optimal concentration of PBM ranged from 2.0 to

5.0 μg/culture. PHA-P was used at 1.25 μl and Con A at 1.25 μg per culture. Various concentrations of pokeweed mitogen (PWM) (Difco Laboratories), ranging from 0.16 to 0.63 μl were added per culture. For assaying antibody-forming cells, a wide range of concentrations of PBM, PWM, and LPS were employed (see Results).

Immunofluorescence. Mouse lymphocytes bearing surface immunoglobulins (sIg) were detected by fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) as described (4). The final dilution of the antisera was 1:10.

Fractionation of Spleen Cells on Nylon Wool Columns. Spleen cells were separated into a nylon-nonadherent, T-enriched fraction and a nylon-adherent, B-enriched fraction on nylon wool (FT-242; Fenwall Laboratories, Inc., Morton Grove, Il.) columns according to the methods of Julius et al. (12) and Handwerger and Schwartz (13) as described (4). Routinely, the nonadherent fraction consisted of 27% and the adherent fraction 55% of the spleen cells loaded onto the column. Viability of both fractions was >97%.

Lysis of Thy 1$^+$ Cells. CBA/J spleen cells were incubated with a 1:8 dilution of anti-Thy 1.2 antisera at 37°C for 60 min, washed once, and then treated with guinea pig complement (diluted 1:8, Grand Island Biological Co.) at 37°C for 30 min. The antisera was prepared by the method of Reif and Allen (14).

Removal of Macrophages. Macrophages were removed either by passage through Sephadex G-10 columns or incubation with carbonyl iron. The efficiency of depletion was monitored by esterase staining and latex ingestion. These procedures have been described in detail previously (4).

Results

Effect of Various Concentration of PBM on Lymphocytes from Spleen, Peripheral Lymph Node, Bone Marrow, and Thymus. 5×10^5 lymphocytes from various lymphoid tissues were cultured with increasing concentrations of PBM in a total volume of 0.25 ml for 48 h. These culture conditions were found to be optimal in preliminary experiments. As seen in Fig. 1., PBM, when used at a concentration as low as 0.125 μg/culture, induced a marked increase in [^{3}H]thymidine incorporation of splenic lymphocytes. Maximal proliferative response was reached when the PBM concentration was increased to 0.5 μg/culture. Further increases in PBM concentrations did not induce

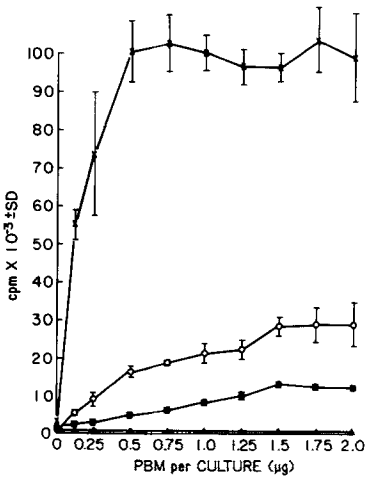

FIG. 1. Mitogenic effect of PBM on lymphocytes from various lymphoid tissues. 5×10^5 lymphocytes from spleen (×), peripheral lymph node (○), bone marrow (■), or thymus (▲) in 0.25 ml were cultured with the indicated concentrations of PBM. At 48 h, triplicate cultures were harvested and [^{3}H]thymidine incorporation expressed as mean counts per minute × 10^{-3} ± SD.

higher levels of proliferation. Peripheral lymph node cells were much less responsive than spleen cells, and the maximal response was obtained only at a higher PBM concentration of 1.5 μg/culture. Bone marrow cells were slightly responsive to PBM whereas thymocytes were totally unresponsive: similar findings were obtained when the time of incubation was extended.

Morphologic studies showed that 60–80% of the viable cells recovered from 48-h spleen cell cultures containing 0.5 μg PBM were blast cells. Moreover, the number of blast cells in PBM cultures correlated well with the level of [^{3}H]thymidine incorporation.

Time-Course of PBM-induced Proliferation of Spleen Cells From CBA/J Mice. Fig. 2 shows that a significant level of [^{3}H]thymidine incorporation occurs in spleen cell cultures containing PBM at 24 h of incubation. When 0.5 μg of PBM was used per culture, the peak proliferative response was at 48 h. Thereafter, the response decreased. In cultures containing suboptimal concentrations of PBM (0.25 μg/culture), the level of [^{3}H]thymidine incorporation also reached a maximum at 48 h, but did not start to decline until after 72 h of incubation. Therefore, 48 h was chosen as the time period of culture for subsequent experiments.

Effect of FCS on the Mitogenicity of PBM. Because the mitogenic effect of PBM is routinely assayed in cultures supplemented with 5% heat-inactivated FCS, it is conceivable that the mitogenic principle is a complex of PBM and serum components. Therefore, the proliferative response of spleen cells both in the presence and absence of serum was examined (Fig. 3). At suboptimal doses of PBM (0.125 and 0.25 μg/culture), the [^{3}H]thymidine incorporation was similar in both types of culture. When higher concentrations of PBM were used, the responses in serum-containing cultures were slightly higher than serum-free cultures. This may be a result of better cell viability in the former. Nevertheless, it is clear that serum components are not necessary for PBM to exert its mitogenic effect.

Response of Lymphocytes from Various Mouse Strains to PBM. To examine whether the activation of lymphocytes by PBM in vitro is a result of earlier exposure to PBM or

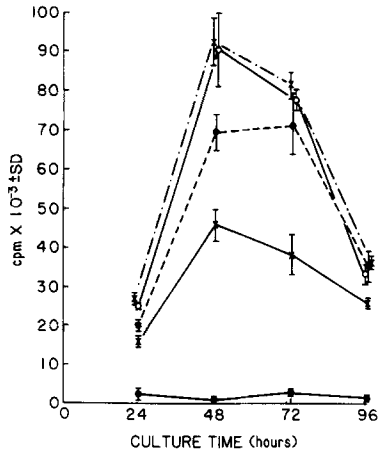

FIG. 2. Time-course of PBM-induced proliferation of CBA/J spleen cells. 5 × 10^5 splenic lymphocytes from CBA/J mice were cultured in the presence of 0.75 μg (×—·—×), 0.5 μg (○——○), 0.25 μg (●----●), or 0.125 μg (×——×) of PBM for various times. Controls contained no PBM (●——●). Indicated are mean counts per minute × 10^{-3} ± SD of triplicate 0.25-ml cultures.

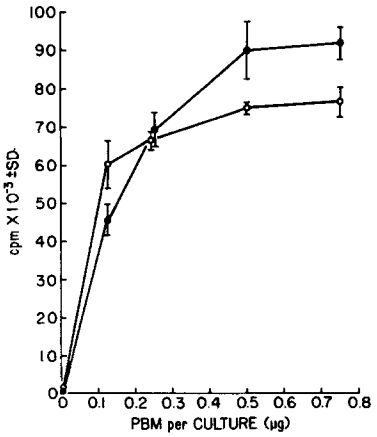

FIG. 3. Effect of fetal calf serum on the stimulation of spleen cells by PBM. Indicated concentrations of PBM were added to 5×10^5 spleen cells and cultured in the presence (●), or absence (○) of 5% fetal calf serum for 48 h. Results are expressed as mean counts per minute $\times 10^{-3} \pm$ SD of triplicate samples.

TABLE I

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

Experiment	Mouse strain	Mitogen/culture				
		PBM, 0.25 µg	PBM, 0.5 µg	Con A, 0.5 µg	LPS*	None
		cpm × 10^{-3} ± *SD*				
1	BALB/c	46.4 ± 2.0	64.1 ± 4.3	—	30.0 ± 3.5	0.9 ± 0
	nu^+/nu^+	51.5 ± 2.6	70.5 ± 8.0	—	34.5 ± 5.1	3.1 ± 0.3
	C_3H/HeJ	24.3 ± 1.1	39.8 ± 2.0	—	9.9 ± 0.3	1.7 ± 0
2	DBA/2J	—	97.7 ± 4.6	154.1 ± 0.4	42.5 ± 2.3	1.5 ± 0.5
	Normal CD-1	106.4 ± 10.8	129.8 ± 0.7	62.3 ± 7.2	98.4 ± 28.0	8.4 ± 1.3
	Axenic CD-1	134.9 ± 12.2	135.7 ± 3.8	54.5 ± 12.9	102.1 ± 12.9	3.6 ± 0.2
	CBA/J	68.7 ± 8.4	85.1 ± 3.9	125.8 ± 9.4	72.0 ± 6.7	2.8 ± 0.8
	CBA/N	43.2 ± 4.8	51.3 ± 7.3	93.8 ± 5.3	34.6 ± 0.6	0.3 ± 0

* LPS in experiment 1 was isolated from *B. pertussis* and was used at 5 µg/culture whereas 1.25 µg of *S. typhosa* LPS from Difco Laboratories was used in experiment 2.

other cross-reactive antigens, the response of spleen lymphocytes from a number of mouse strains was assayed (Table I). Lymphocytes from normal inbred mouse strains, such as BALB/c, DBA/2J, and CBA/J responded well to PBM, as did outbred CD-1 animals. Moreover, axenic CD-1 mice were as reactive as their counterparts raised under conventional conditions. Therefore, PBM is a nonspecific mitogen for mouse lymphocytes.

It is interesting to note that three mouse strains with known defects in immune functions also responded to PBM. First, spleen cells from athymic nude mice of BALB/c background proliferated to a similar extent as cells from BALB/c mice indicating that the proliferating cells in PBM cultures were not T lymphocytes. Moreover, the stimulation of lymphocytes by PBM could occur in the absence of T cells. Secondly, C3H/HeJ mice, which were low responders to the mitogenic effects of LPS (15), failed to respond to LPS isolated from *B. pertussis,* but were markedly

activated by PBM. This suggested that the mitogenic principle in PBM was probably not LPS. Thirdly, when CBA/N mice with a known defect in the maturation of B lymphocytes were used (16), the [^{3}H]thymidine incorporation of their splenic lymphocytes was approximately 60% that of cells from normal CBA/J mice. However, the background incorporation of cultures containing no mitogen was also much lower than that of CBA/J cultures. Therefore, it appeared that the B cell defect in CBA/N mice had little effect on the proliferation of spleen cells after PBM stimulation.

Nature of the Proliferating Lymphocytes. The finding that spleen cells from congenitally athymic nude mice were responsive to PBM suggested that the lymphocytes stimulated by PBM were not T cells. To delineate further the nature of cells activated by PBM, spleen cells from CBA/J mice were cultured with 0.5 μg PBM for 48 h, recovered, and stained for sIg by fluorescein-conjugated F(ab')$_2$ fragments of rabbit anti-mouse IgG. 60–80% of the viable cells recovered were blast cells, and >95% of these blasts were sIg$^+$ (Table II). Electron microscopic examination of blasts also revealed morphology of plasmablasts with dilated vesicular rough endoplasmic reticulum as described by Janossy et al. (17). Furthermore, when nylon-separated spleen cells were cultured with PBM, a proliferative response, similar in magnitude to that of unseparated cells, was only found associated with the nylon-adherent fraction containing >85% sIg$^+$ cells (Table III).

PBM-induced Proliferation of Lymphocytes in the Absence of T Cells. To confirm further that the response of B lymphocytes to PBM is T cell independent, spleen cells were

TABLE II

Detection of sIg on PBM-stimulated Spleen Cells

Cells/culture*	Mitogen	Cells recovered at 48 h		
		Small lymphocytes	Blasts	sIg$^+$ blasts
× 10^6			× 10^6	
20	PBM, 2 μg/ml	2.1	8.4 (80)‡	8.0
20	LPF, 2 μg/ml	2.2	5.6 (72)	0.2
20	None	3.6	0.3 (8)	0.2

* 4 × 10^6 spleen cells in 2 ml were cultured in tubes with or without mitogens. At 48 h, cells from five tubes were pooled and stained with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-mouse IgG as described in Materials and Methods.

‡ Number in parenthesis represents the percentage of viable cells with morphology of blasts.

TABLE III

Proliferative Responses of Spleen Cells Separated on Nylon Wool Columns

Spleen cell	Mitogen/culture			
	PHA, 0.18 μl	Con A, 0.5 μg	PBM, 0.5 μg	None
Unseparated	21.8 ± 2.7*	145.8 ± 5.1	146.1 ± 6.6	7.9 ± 0.7
Nonadherent	18.8 ± 1.0	146.1 ± 15.3	1.7 ± 0.1	0.1 ± 0.1
Adherent	0.8 ± 0.1	4.6 ± 0.4	139.9 ± 1.5	0.7 ± 0.4

* Counts per minute × 10^{-3} ± SD of triplicate cultures of 0.5 × 10^6 CBA/J lymphocytes in 0.25 ml at 48 h.

treated with Thy-1 antiserum and complement before culture. Table IV indicates that such treatment abolished the Con A response but had no effect on stimulation by the B cell mitogen, LPS, or by PBM. This finding, together with the fact that spleen cells from adult thymectomized, X-irradiated mice reconstituted with fetal liver cells (TxB mice) were responsive to PBM (Table V), suggested that PBM was mitogenic for mouse B lymphocytes even in the absence of T cells.

Effect of Macrophage Depletion on the Proliferative Response of Spleen Cells. Macrophages have been implicated as accessory cells for some mitogens (18–21). To delineate their role in PBM-induced proliferation, spleen cells were cultured before and after macrophages were removed by either passage through Sephadex G-10 or incubation with carbonyl iron. As monitored by esterase staining and latex ingestion, <1% macrophages were found in spleen cell populations treated by either procedure. Nevertheless, treated spleen cells responded to PBM as well as their untreated counterparts (Table VI). Hence, macrophages are not required for the activation of B cells by PBM.

Failure of Anti-LPF Serum to Inhibit the Mitogenic Effects of PBM. Because PBM and LPF were separated from culture supernatant fluids of *B. pertussis* by molecular sieving, it was possible that PBM was an aggregated form of LPF or that it contained the mitogenic principle of LPF. Indeed Con A and PHA, both T mitogens, have been shown to stimulate B lymphocytes when presented in insolubilized forms (22, 23). To

TABLE IV

Effect of T Cell Depletion on the Mitogenic Response of CBA/J Spleen Cells to PBM

Treatment	Mitogen/culture				
	PBM, 0.25 μg	PBM, 0.5 μg	Con A, 0.5 μg	LPS, 1.25 μg	None
None	94.1 ± 15.7*	91.3 ± 16.2	99.3 ± 18.0	80.5 ± 8.9	3.4 ± 0.4
Anti-Thy-1 plus GPC	73.9 ± 4.9	82.3 ± 3.3	0.4 ± 0	73.9 ± 8.4	0.6 ± 0.3
Anti-Thy-1	91.2 ± 7.9	91.9 ± 1.4	91.1 ± 5.4	82.1 ± 14.8	ND
GPC	75.4 ± 1.9	90.0 ± 14.2	56.4 ± 3.3	67.2 ± 4.0	ND

CBA/J spleen cells were treated with Thy-1.2 antiserum and guinea pig complement (GPC) as described in Methods. Controls are spleen cells treated with either Thy 1.2 antiserum only or GPC only. ND, not done.

* Counts per minute × 10^{-3} ± SD of triplicate cultures of 5 × 10^5 lymphocytes at 48 h.

TABLE V

PBM-induced Proliferation of Spleen Cells from TxB Mice

Mitogen	Source of spleen cells	
	CBA/J	TxB
PBM, 0.5 μg	143.5 ± 3.6	113.1 ± 6.7
ConA, 0.5 μg	130.9 ± 6.4	19.1 ± 4.2
LPS, 1.25 μg	124.3 ± 4.8	96.2 ± 2.8
None	7.9 ± 2.4	5.6 ± 0.5

TxB mice were 6-wk-old CBA/J animals, which had been adult thymectomized, X-irradiated, and reconstituted with 14- to 16-d-old fetal liver cells. 5 × 10^5 spleen cells from these mice were cultured in the presence of the indicated mitogens for 48 h. Controls were spleen cells from normal CBA/J mice. Indicated are the mean counts per minute × 10^{-3} ± SD of triplicate samples.

TABLE VI

Effect of Macrophage Depletion on the Mitogenic Response of CBA/J Spleen Cells to PBM

Experiment	Treatment	Mitogen/culture			
		PHA-P, 0.18 μl	Con A, 0.5 μg	PBM, 0.5 μg	None
		cpm × 10^{-3} ± *SD*			
1	None	21.8 ± 2.7*	145.8 ± 5.1	146.1 ± 6.6	7.9 ± 0.7
	G-10 column	28.3 ± 1.2	103.2 ± 8.8	166.5 ± 8.1	1.8 ± 0.5
2	None	ND	242.5 ± 21.2	131.7 ± 6.5	8.6 ± 1.7
	Carbonyl-iron	ND	206.3 ± 8.9	148.4 ± 16.8	2.1 ± 0.2

* [^{3}H]thymidine incorporation of 5 × 10^5 CBA/J spleen cells at 48 h.

TABLE VII

Failure of Monospecific Anti-LPF Serum to Inhibit PBM-induced Mitogenesis of CBA/J Spleen Cells

Serum added per culture	Mitogen/culture						
	LPF, 0.25 μg	LPF, 0.5 μg	PBM, 0.25 μg	PBM, 0.5 μg	Con A, 0.5 μg	LPS, 1.25 μg	None
None	17.1 ± 3.2	34.1 ± 2.9	98.6 ± 0.5	94.3 ± 11.9	55.0 ± 5.7	80.0 ± 10.6	1.7 ± 0.1
Anti-LPF, 250 nl	3.5 ± 0.8	6.5 ± 0.3	95.4 ± 2.2	109.6 ± 4.0	55.8 ± 8.1	76.3 ± 1.9	4.4 ± 1.0
Anti-LPF, 625 nl	3.6 ± 0.2	3.6 ± 0.7	97.6 ± 6.0	110.8 ± 18.1	80.7 ± 8.7	79.7 ± 5.1	4.3 ± 0.4
Anti-LPF, 1,250 nl	ND	2.8 ± 0.6	ND	87.9 ± 9.7	58.4 ± 2.4	77.4 ± 4.3	2.0 ± 0.3
NRS, 250 nl	14.0 ± 1.5	26.7 ± 2.6	106.8 ± 12.9	122.4 ± 7.0	62.1 ± 0.8	83.9 ± 7.4	2.8 ± 0.4
NRS, 625 nl	15.3 ± 2.8	44.2 ± 6.0	120.4 ± 11.8	119.1 ± 9.9	89.6 ± 6.9	82.9 ± 0.7	3.5 ± 0.3
NRS, 1,250 nl	ND	37.5 ± 2.7	ND	90.2 ± 6.0	ND	ND	4.8 ± 1.7

5 × 10^5 CBA/J spleen cells were cultured in the absence or presence of indicated amounts of mitogen and sera for 48 h. Indicated are counts per minute × 10^{-3} ± SD of triplicate cultures. ND, not done.

test whether PBM mitogenic activity was related to LPF, monospecific anti-LPF serum (1) was added to PBM cultures (Table VII). It is clear that as little as 250 nl of anti-LPF serum totally abolished the LPF response, but failed to affect PBM mitogenic activity. Similar findings were obtained when the concentration of antiserum was increased to 1,250 nl/culture. Therefore, it appeared that the mitogenic principle of PBM is distinct from that of LPF.

Polyclonal Activation of CBA/J Spleen Lymphocytes by PBM. In a humoral immune response, B lymphocytes with specificity for the relevant antigen will proliferate and then differentiate to mature antibody-forming cells (AFC). After the activation of B lymphocytes by some mitogens, these cells not only undergo DNA synthesis but also produce and secrete antibodies of various specificities. These AFC can be detected by a modified plaque assay (7). When spleen cells cultured with PBM for 48 h were recovered and assayed for AFC, it was found that cells secreting 19S IgM antibodies against sheep erythrocyte antigens and an unrelated hapten TNP were present (Table VIII). Therefore, PBM is also a polyclonal activator of mouse B cells.

Mitogenic Effects of PBM on HPBL. To determine whether PBM is also a mitogen for human lymphocytes, 0.125 × 10^6 HPBL were cultured with various concentrations of PBM in 0.25 ml. These culture conditions were determined by preliminary experiments. Table IX shows the [^{3}H]thymidine incorporation of HPBL from nine normal healthy adults in the presence of PBM. All nine individuals were responsive to PBM; the optimal mitogenic concentrations ranged from 2 to 5 μg/culture. Most data obtained thus far were from cultures supplemented with 5% FCS. However,

TABLE VIII

Polyclonal Activation of CBA/J Spleen Cells by PBM

Mitogen/culture	Viable cells recovered × 10^6*	Direct PFC/10^6 viable cells	
		SRBC	TNP-SRBC
PBM			
1 μg	6.4	21	582
2 μg	6.9	25	532
3 μg	5.4	34	753
4 μg	6.9	34	401
LPS			
5 μg	5.3	38	776
10 μg	6.2	20	766
20 μg	5.5	38	648
30 μg	8.6	37	507
LPF			
1 μg	6.9	0	10
2 μg	7.2	0	6
5 μg	7.0	0	9
None	4.8	2	56

* Pooled from duplicate cultures containing 10 × 10^6 spleen cells in 1 ml.

TABLE IX

Proliferation of Human Peripheral Blood Lymphocytes in Response to PBM

Mitogen/culture	Donors								
	C. S.	S. K.	S. M.	J. B.	J. G.	W. L.	M. H.	C. M.	C. M.*
PBM, 1.5 μg	72.8 ± 5.2‡	43.2 ± 3.4	ND	ND	ND	44.8 ± 3.5	ND	ND	ND
PBM, 2.0 μg	128.6 ± 9.2	80.7 ± 10.7	98.9 ± 6.2	48.5 ± 5.3	ND	58.8 ± 5.3	ND	29.6 ± 2.6	ND
PBM, 2.5 μg	131.9 ± 9.4	82.4 ± 3.2	ND	63.8 ± 11.7	107.9 ± 10.2	90.0 ± 3.9	96.9 ± 9.4	81.6 ± 7.5	57.8 ± 4.5
PBM, 5.0 μg	ND	ND	ND	72.2 ± 9.9	ND	ND	ND	81.7 ± 9.3	62.9 ± 8.1
Con A, 1.25 μg	113.0 ± 8.1	61.1 ± 0.7	59.6 ± 5.8	ND	ND	76.0 ± 11.0	73.8 ± 6.2	ND	ND
PHA-P, 1.25 μl	203.1 ± 2.4	131.4 ± 5.4	225.9 ± 17.8	ND	216.6 ± 4.7	95.1 ± 4.9	147.0 ± 8.3	ND	ND
PWM, 0.16 μl	ND	ND	ND	90.1 ± 10.8	ND	ND	ND	82.9 ± 14.4	ND
PWM, 0.31 μl	ND	ND	ND	86.7 ± 10.5	ND	ND	ND	87.0 ± 10.4	105.3 ± 5.3
PWM, 0.63 μl	ND	ND	ND	70.9 ± 9.9	47.1 ± 7.4	ND	ND	76.9 ± 4.6	67.7 ± 5.5
None	0.6 ± 0.1	0.1 ± 0	0.2 ± 0	2.4 ± 0.8	3.2 ± 1.0	0.5 ± 0.1	4.6 ± 1.5	1.2 ± 0.7	0.6 ± 0.3

* Culture supplemented with 5% pooled AB serum instead of 5% FCS.

‡ Counts per minute × 10^{-3} ± SD of triplicate cultures containing 1.25 × 10^5 HPBL in 0.25 ml at 120 h. ND, not done.

HPBL were also responsive when cultured in the presence of 5% pooled human AB serum. Time-course studies showed that maximum stimulation occurred after 4–6 d of culture. Therefore, all cultures were harvested at 120 h. Studies were also carried out to examine the morphology of HPBL after stimulation by optimal concentrations of PBM for 5 d. When examined after Wright-Giemsa staining, 40–55% of the viable cells recovered were found to be blast cells.

Proliferation of Human Cord Blood Lymphocytes in Response to PBM. The fact that all

normal adults tested were responsive to the in vitro stimulation by PBM strongly suggested that PBM was a nonspecific mitogen. To study further this notion, the mitogenic effect of PBM on cord blood lymphocytes was examined. It was found that cord blood lymphocytes could be induced to proliferate by PBM (Table X). Therefore, it appears that the proliferation observed in PBM cultures is not a result of antigenic stimulation of presensitized lymphocytes but rather is caused by nonspecific activation of PBM.

PBM is a Polyclonal Activator of HPBL. Because PBM-stimulated mouse B lymphocytes produce polyclonal antibodies in vitro, the effect of PBM on HPBL antibody formation was examined. The detection of human plaque-forming cells (PFC) after stimulated by PWM have been well studied by Fauci and Pratt (10). Therefore, similar procedures were used for PBM cultures. 2×10^6 HPBL were cultured with various concentrations of PBM in media supplemented with 10% pooled AB serum. After 6 d of culture, the cells were recovered and assayed for antibodies against sheep red blood cells (SRBC) and SRBC coated with TNP. As seen in Table XI, these antibodies were secreted by PBM-cultured HPBL leading to plaque formation in the hemolytic plaque assay. However, PBM-induced antibody formation differed in several aspects form the mitogenic stimulation by PBM. Firstly, a much higher concentration of PBM was required for polyclonal activation (ranging from 20 to 40 μg/culture) as compared with that needed for proliferative responses (2–5 μg/culture). This may be, in part, accounted for by the difference in cell numbers and culture conditions in the two assay systems. Secondly, in contrast with proliferation, different individuals vary greatly in their response to the polyclonal activation by PBM. Thirdly, the range for optimal stimulation of lymphocytes from a given individual tend to be much narrower in the PFC assay than for [^{3}H]thymidine incorporation. Lastly, maximum numbers of PFC were detected on day 6 of culture whereas little or no PFC could be found on days 5 or 7. For proliferative responses even though the optimal culture period was usually 5 d; the decrease in response on day 4 or 6, was not as marked as that in the PFC assay.

Discussion

In addition to the T cell mitogen, LPF, culture supernatant fluids of phase I *B. pertussis* also contains a mitogen and polyclonal activator for mouse B lymphocytes. It

TABLE X

Mitogenic Effects of PBM on Cord Blood Lymphocytes

Mitogen/culture	Counts per minute × 10^{-3} ± SD*
PBM, 2 μg	3.9 ± 0.9
PBM, 3 μg	11.8 ± 0.8
PBM, 4 μg	36.0 ± 5.0
PBM, 5 μg	59.2 ± 4.7
Con A, 0.625 μg	48.9 ± 1.6
Con A, 1.25 μg	40.2 ± 2.9
PHA-P, 0.625 μl	115.8 ± 6.4
PHA-P, 1.25 μl	98.7 ± 9.2
None	2.5 ± 0.5

* From triplicate cultures containing 2.5×10^5 cord blood lymphocytes harvested at 120 h. The culture medium was supplemented with 5% FCS.

TABLE XI

Polyclonal Activation of Human Peripheral Blood Lymphocytes by PBM

Mitogen	Donor 1			Donor 2			Donor 3	
	Viable cells recovered $\times 10^6$*	SRBC PFC/ 10^6 cells	TNP-SRBC PFC/ 10^6 cells	Viable cells recovered $\times 10^6$	SRBC PFC/ 10^6 cells	TNP-SRBC PFC/ 10^6 cells	Viable cells recovered $\times 10^6$	SRBC PFC/10^6 cells
PWM								
30 μl	1.7	163	402	1.8	51	36	1.2	60
20 μl	2.1	150	192	1.4	98	179	1.0	0
10 μl	3.6	114	98	2.0	18	33	0.9	89
5 μl	3.4	122	122	1.8	134	150	1.4	0
2.5 μl	2.6	104	166	2.2	19	57	2.8	8
1.25 μl	1.6	60	217	2.2	65	73	2.9	0
PBM								
70 μg	1.4	20	15	0.7	24	7	1.6	3
60 μg	1.5	0	9	0.4	17	0	2.1	82
50 μg	1.6	4	4	0.8	5	8	1.4	90
40 μg	1.1	8	55	0.8	22	22	0.8	0
30 μg	1.4	205	216	0.7	129	96	ND	ND
20 μg	1.7	192	273	0.6	155	79	ND	ND
None	0.9	2	0	0.4	5	0	0.6	0

* Pooled from triplicate cultures of 2×10^6 HPBL in 1 ml at 120 h. ND, not done.

is clear that only B cells will proliferate in response to this mitogen in vitro because >95% of the blast cells recovered from 48 h cultures bear sIg. Hence, this mitogen is termed the PBM.

The responding B cells are adherent to nylon-wool. Moreover, they can proliferate in response to PBM in the absence T cells because (*a*) spleen cells from nu^+/nu^+ or TxB mice are responsive to PBM, and (*b*) pretreatment of spleen cells with Thy-1 antiserum and complement has little effect on their PBM-induced proliferation. Macrophages also seem to play little role in the mitogenic effect of PBM because macrophage depletion by either passage through Sephadex G-10 or incubation with carbonyl iron fails to decrease the [^{3}H]thymidine incorporation of PBM cultures. As in the case of many B cells mitogens (24–26), PBM also induces the differentiation of mouse splenic B cells into AFC. However, whether this property of PBM is also completely independent of T cells and macrophages is not certain.

Thus far, all mouse strains tested, including germfree CD-1 animals, are responsive to PBM. Furthermore, HPBL from all donors as well as cord blood lymphocytes are induced to synthesize DNA by PBM. Therefore, PBM seems to activate lymphocytes nonspecifically.

Despite the occurrence of both in the same protein peak excluded from Sephadex G-150, PBM and the HA of *B. pertussis* seem to be associated with distinct moieties. It is possible to remove all the HA activity by extensive adsorption with sheep erythrocyte stroma (ghost) without affecting the mitogenicity of the fraction (M. Ho. Unpublished observations.). Preliminary analysis by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME) indicate that the excluded peak contains five major polypeptides. However, after adsorption with erythrocyte stroma, only two remained. Whether one or both

these polypeptides or repeating units of them, are the mitogenic prinicple(s) of PBM is not known.

It has been shown that Con A and PHA, though mitogenic for T cells when soluble, are capable of stimulating B lymphocytes when presented in insoluble forms. (22, 23). Because LPF and PBM are isolated from culture supernatant fluids of *B. pertussis* by similar procedures, it is conceivable that the B cell mitogenic activity observed is merely produced by aggregates of LPF or a complex containing LPF components. Therefore, the effects of a monospecific rabbit anti-LPF serum on PBM mitogenesis was explored. This antiserum failed to diminish the mitogenic activity of PBM even when used at five times the concentration required to block LPF-induced proliferation. Furthermore, double immunodiffusion studies employing both monospecific anti-LPF serum and a heterospecific antiserum raised against killed *B. pertussis* organisms indicate that LPF is immunologically distinct from PBM (data not shown). Moreover, SDS-PAGE of LPF in the presence of 2-ME shows four major polypeptides, none of which is found in the PBM fraction before or after adsorption with sheep erythrocyte stroma.

There is ample evidence suggesting that the LPS of many Gram-negative bacteria is a mitogen as well as polyclonal activator for mouse B lymphocytes. However, the mitogenic effect of PBM is not a result of LPS contamination based on experiments with C3H/HeJ mice, a known low responder to the in vitro activation by LPS. Spleen cells from these mice indeed respond poorly to LPS isolated from *B. pertussis* but proliferate normally in the presence of PBM.

Preliminary results show that most of the blast cells in PBM cultures of HPBL possess intracellular Ig. However, more stringent cell fractionation and marker studies are required before the nature of PBM-responsive cells are well defined. There is no doubt that at least some human peripheral B cells are activated by PBM because only B cells can be induced to produce antibody. In view of the fact that the stimulation of mouse B cells by PBM is independent of T cells or macrophages, this is likely to be the case for human B lymphocytes also. If this notion is confirmed, PBM can be an excellent probe for the evaluation of human B lymphocytes function because of the high [^{3}H]thymidine incorporation and PBM cultures. Indeed, the level of incorporation is much higher than that induced by the known specific human B cell mitogens, namely, *Nocardia* water-soluble mitogen (NWSM) (27), and endotoxin protein (EP) isolated from Gram-negative bacteria (28). Both of these mitogens are also polyclonal activators for human B cells. However, it is unlikely that PBM is related to either of these substances. In the case of NWSM, extremely weak polyclonal-activating effects can be detected. Moreover, PBM differs from EP in several respects. Firstly, in contrast with PBM-induced mitogenesis, the proliferation of EP cultures of HPBL is totally suppressed by the presence of human AB serum. Secondly, the peak of EP responses is at day 3 whereas that for PBM is usually at day 5. Thirdly, much lower concentrations of PBM are required for the induction of DNA synthesis (2–5 μg of protein per culture) as compared with EP (25–50 μg/culture). Lastly, the mitogenic proteins in *S. typhosa* EP seem to have molecular weights lower than 17,000 (29), whereas PBM obtained after adsorption with sheep erythrocyte stroma is comprised of two major polypeptides with molecular weights of 18,500 and 58,000. However, firm conclusions cannot be drawn until a comparison is made between PBM and EP of *B. pertussis*.

B. pertussis organisms are widely used as adjuvants in the production of IgG and IgM antibodies, reaginic antibody, and experimental allergic encephalomyelitis (reviewed in reference 30). LPS of *B. pertussis* can probably account for some, but not all, of the adjuvant effects of the whole organism. Thus, LPF has been implicated as an adjuvant for reaginic antibody formation (31), but not for other antibody classes. Dresser (32) showed that at high doses of antigen, *B. pertussis* caused an increase in IgM antibody production by acting directing on B cells. Because PBM can induce polyclonal IgM formation in vitro, it will be of interest to investigate its adjuvanticity both in vitro and in vivo.

Summary

Pertussis B mitogen (PBM), isolated from culture supernatant fluids of *Bordetella pertussis,* is a potent mitogen for mouse and human lymphocytes. In mice, >95% of the blast cells recovered from PBM cultures bear surface immunoglobulins. Therefore, PBM seems to induce proliferation of mouse B lymphocytes, but not T cells. The proliferative response observed is nonspecific because cells from all mouse strains tested, including germfree animals, are responsive. Moreover, the mitogenic activity of PBM is independent of T lymphocytes, macrophages, or serum factors. When human peripheral blood or cord blood lymphocytes are cultured in the presence of PBM, a high level of thymidine incorporation by these cells is detected. Furthermore, PBM can induce polyclonal antibody formation by both mouse and human lymphocytes. Despite similar methods of isolation, PBM is distinct from the lymphocytosis-promoting factor of *B. pertussis,* a previously described T cell mitogen.

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