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

III. B-Cell Dependence for T-Cell Proliferation*

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Lymphocytosis-promoting factor (LPF)[1], purified from culture supernatant fluids of phase I *Bordetella pertussis*, produces several effects in vivo including: (*a*) leukocytosis with a predominant lymphocytosis; (*b*) sensitization to histamine; and (*c*) hypoglycemia and unresponsiveness to the hyperglycemic effect of epinephrine (1). The lymphocytosis is not the result of cellular proliferation, but of the inability of circulating lymphocytes to home from the blood into lymphoid tissues at a normal rate (2, 3).

Although LPF does not cause proliferation of lymphocytes in vivo, murine and human lymphocytes are stimulated in vitro by LPF (4–7). LPF is mitogenic for lymphocytes from the spleen and lymph nodes of mice but bone marrow lymphocytes and normal thymocytes are unresponsive. Cells from the thymus of cortisone-treated mice, however, are stimulated by LPF. When spleen or lymph node cells are pretreated with Thy-1 antiserum plus complement, the LPF response is abolished. Moreover, after 72 h of culture with LPF, the stimulated cells have the electron microscopical appearance of T-cell blasts, and addition of Thy-1 antiserum and complement at this time destroys the cells that incorporate ^{3}H-thymidine. Taken together, these findings show that LPF is a T-cell mitogen.

However, when CBA spleen or lymph node cells are fractionated on nylon wool columns, neither the T-cell-enriched nonadherent nor the B-cell-enriched adherent fraction responds optimally to LPF. Full response is restored, however, when these fractions are cultured together (5). Hence, the stimulation of murine T lymphocytes by LPF requires the presence of nylon wool-adherent accessory cells. Current studies in this laboratory are designed to elucidate the nature and function of the cells that modulate the mitogenic effect of LPF on mouse T cells. The findings reported herein define the properties and nature of the accessory helper cells and demonstrate that they are cells with surface immunoglobulins.

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[1] *Abbreviations used in this paper*: A, nylon wool-adherent spleen cells; BSA, bovine serum albumin; CR, complement-receptor; Con A, concanavalin A; EA, sheep erythrocytes sensitized with anti-sheep antibodies; EAC, sheep erythrocytes sensitized with anti-sheep erythrocyte antibody and mouse C3; FACS, fluorescence-activated cell sorter; FcR, receptor for Fc portion of immunoglobulin; FCS, fetal calf serum; FeC, carbonyl iron; LPF, lymphocytosis-promoting factor; LPS, lipopolysaccharide; NA, nylon wool-nonadherent spleen cells; PBS, phosphate-buffered saline; PHA-P, phytohemagglutinin-P; RaMIg, rabbit anti-mouse immunoglobulin; sIg, surface immunoglobulin; SRBC, sheep erythrocytes; VBG, veronal buffer with gelatin.

Materials and Methods

Animals. Female CBA/J mice, obtained from The Jackson Laboratories (Bar Harbor, Maine) or from Jackson stock bred in this department, were employed.

Medium. The medium used for preparation of cell suspensions and for cultures was RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% fetal calf serum (FCS) (Microbiological Associates, Walkersville, Md.), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cell Suspensions. Single cell suspensions from the spleen, thymus, peripheral lymph nodes, and bone marrow of adult mice were prepared as described previously (4).

Suspensions of single cells from all organs were washed once, resuspended in medium, and viable cells were enumerated using the trypan blue dye exclusion test. Only preparations containing >90% viable cells were used.

Fractionation of Lymphoid Cells. Spleen cells were separated into nylon wool adherent, B-cell-enriched, and nonadherent, T-cell-enriched populations on nylon wool (FT-242, Fenwall Laboratories, Inc., Morton Grove, Ill.) columns by the methods of Julius et al. (8) and Handwerger and Schwartz (9). All operations were performed at 37°C. 300 × 10^6 spleen cells in 4 ml of medium were loaded on a 12-ml column in a plastic 20-ml syringe, followed by 4 ml of medium. After 45 min in a humidified incubator, 15 ml of nonadherent cells were collected at a rate of 1 ml/2–3 min. The nylon wool in the column was washed with 100 ml of medium and then teased with forceps. Adherent cells were released by five cycles of compression with a syringe plunger using 10 ml of medium for each compression. Usually, 27% of the cells loaded onto the column were collected in the nonadherent fraction whereas 55% were in the adherent fraction. Viability in both fractions was always >97%.

Nylon wool-adherent cells were separated on discontinuous bovine serum albumin (BSA) density gradients using the methods of Raidt et al. (10) and Adler et al. (11). 35.3% BSA (Path-o-cyte 4, Miles Laboratories, Inc., Ames Div., Elkhart, Ind.) was diluted appropriately in RPMI-1640 and 3.5-ml layers of 32, 29, 26, and 23% BSA were placed in a 17-ml cellulose nitrate tube. 600 × 10^6 lymphocytes in 3 ml of 10% BSA were layered on top of the gradient and the tubes were centrifuged at 17,500 *g* for 30 min at 4°C in an SW 27 rotor, Beckman Instruments, Palo Alto, Calif. Discrete bands of cells which formed at the interfaces were collected and washed extensively with RPMI-1640. Viability of cells after centrifugation was always >98%, whereas total recovery ranged from 80 to 93%.

Cytolysis by Anti-Thy-1.2 Antisera. Anti-Thy-1.2 antisera, kindly supplied by Dr. M. Howe, was prepared by the method of Reif and Allen (12). Cytolysis of Thy-1.2 positive cells was performed as follows. 25 × 10^6 CBA lymphocytes were incubated in 1 ml of antisera (diluted 1:8 with RPMI-1640-5% FCS) for 60 min at 37°C. The cells were then washed once, resuspended in a 1:8 dilution of guinea pig complement (Grand Island Biological Co.) previously absorbed with normal CBA spleen cells, and incubated for 30 min at 37°C. Subsequently the cells were washed twice and viability determined by trypan blue exclusion. Control cells were treated with antisera alone or complement alone.

Mitomycin-C Treatment. Mitomycin-C (Sigma Chemical Co., St. Louis, Mo.) was diluted to 1 mg/ml in medium immediately before use. 0.35 ml was added per 10 ml of lymphocyte suspension containing 20 × 10^6 cells. The suspensions were then incubated at 37°C for 1 h and the cells washed three times before culture.

Mitogens. LPF was isolated from Phase I *Bordetella pertussis* strain National Institutes of Health 114 as previously described (1). Dilutions were made in medium from a stock solution of LPF at 100 μg/ml in 4 M urea −0.1 M PO_4-1 M NaCl, pH 6.4.

Concanavalin A (Con A, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was reconstituted with sterile distilled water and dispensed into 0.2-ml aliquots at 400 μg/ml. Phytohemagglutinin-P (PHA-P) was purchased from Difco Laboratories, Detroit, Mich. and stored in aliquots. In earlier experiments lipopolysaccharide (LPS) was kindly provided by Dr. B. M. Sultzer of this department and was prepared from *Salmonella typhosa* by phenol-water extraction (13, 14). In later experiments, Difco *S. typhosa* LPS (phenol-water preparation) (Difco Laboratories) was employed.

All mitogens were usually used at optimal doses: Con A, 0.5 μg; PHA-P, 0.18 μl; LPS, 1.25 μg (Difco Laboratories) or 2.5 μg (Sultzer); and LPF, 0.5 μg per culture.

Cell Cultures and Incorporation of ^{3}H-Thymidine. 0.25 ml of cell suspensions containing 0.5 × 10^6, or the appropriate number of cells, were dispensed into wells of sterile flat-bottom microtiter plates (model IS-FB096TC, Linbro Chemical Co., Hamden, Conn.). Triplicate cultures were incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air for 72 h unless otherwise stated. 8 h before harvest, 2 μCi of ^{3}H-thymidine in 0.02 ml (sp act 5 Ci/mM, TRA 120, Amersham Corp., Arlington Heights, Ill.) was added to each well. Cells were harvested on glass fiber filter papers (grade 934H, Reeve Angel, Clifton, N.J.) using an automated cell collector (Otto Hiller Co., Madison, Wis.) and washed with saline. The filters were dried overnight and placed into scintillation vials. 3 ml of an Omnifluor-toluene scintillation mixture (4 g/liter, New England Nuclear, Boston, Mass.) were dispensed into each vial and the samples then counted in a liquid scintillation counter. The proliferative response was usually expressed as the net mean counts per min (cpm) × 10^{-3} of triplicate cultures ± 1 SD.

Lysis of sIg$^+$ Lymphocytes. Rabbit anti-mouse Ig (RaMIg, N. L. Cappel Laboratories Inc., Cochranville, Pa.) was absorbed with CBA thymuses (1 thymus/0.2 ml antiserum) two times for 1 h at 40°C. 20 × 10^6 lymphocytes were incubated with 1 ml of RaMIg (1:50) for 20 min at 4°C, pelleted and resuspended in an equal volume of a 1:2 dilution of rabbit complement (N. L. Cappel Laboratories Inc.) previously twice absorbed with CBA spleens (1 spleen/0.7 ml complement). Tubes were incubated first at 4°C for 10 min, then at 37°C for 20 min. Dead cells were removed from the cell suspension by centrifugation through a Ficoll-Conray gradient (Ficoll was purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. and Conray from Mallinckrodt Pharmaceuticals, St. Louis, Mo.) (15) and lysis of sIg$^+$ cells was monitored by immunofluorescence.

Sheep Erythrocytes Sensitized with Anti-Sheep Erythrocyte Antibody and Mouse C3 (EAC) Rosettes. Cells bearing complement receptors (CR) were rosetted by the method of Bianco (16). Briefly, EA were prepared by incubating a 5% suspension of sheep erythrocytes (SRBC) in Hank's balanced salt solution with an equal volume of IgM rabbit anti-SRBC (1:100, Cordis Laboratories Inc., Miami, Fla.) for 30 min at 37°C. After washing two times, the EA complexes were resuspended in veronal-buffer with gelatin (VBG) to 5%. An equal volume of a 1:4 dilution of C5-deficient mouse serum (collected from DBA mice maintained at Downstate Medical Center) was then added to the EA and the mixture incubated for 20 min at 37°C to form EAC. The reaction was stopped by adding cold VBG to the mixture. EAC were washed three times and resuspended to 5%. Lymphocytes at 15 × 10^6 cells/ml were then mixed with 1 ml of 2% EAC for 30 min at 37°C. The rosetted (CR$^+$) cells were separated from the nonrosetting ones by centrifugation through a Ficoll-Conray gradient (2,000 *g* for 20 min at 20°C) by the procedures of Parish (17). The CR$^+$ cells were freed from the rosettes by lysis of erythrocytes with tris-NH_4 Cl. About 30–35% of spleen cells were rosetted by this procedure and rosettes were not detected when EA were used.

Cell Separation on the Fluorescence-Activated Cell Sorter (FACS). Spleen cells were stained by a two-step protocol: first with RaMIg, then fluoresceinated-goat anti-rabbit Ig. Stained cells were analysed for the percentage of bright fluorescent cells and sorted on a FACS (Becton, Dickson Electronics Laboratory, Mountain View, Calif.) as described (18, 19). The bright cell fraction contained at least 98% bright cells whereas the dull cells were contaminated with up to 4% bright cells based on repeated analysis of sorted cells. Flow rates were ≅ 800 cells/s for analysis and 4,000 cells/s for separation.

Separation of Surface Immunoglobulin-Positive (sIg$^+$) Lymphocytes. Separation of sIg$^+$ cells on an anti-Ig glass bead column was modified from the method of Wigzell (20). Glass beads (Superbrite, 090-5005, diameter 200–225 μm, 3M Co., St. Paul, Minn.) were acid cleaned as follows. They were first soaked in a 1:1 mixture of concentrated sulfuric acid, rinsed in tap water for 24 h, soaked in a large volume of a dilute solution of sodium carbonate for 24 h, rinsed repeatedly in glass-distilled water, soaked in 1% HCl, rinsed repeatedly in glass-distilled water, dried, and autoclaved. A 1.5 × 30-cm glass column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.), sterilized by treating with 70% ethanol overnight was used to hold the beads. The beads were first coated with mouse Ig, prepared by ammonium sulfate precipitation, by incubating them with 1 mg/ml or more of Ig overnight at 4°C. They were then washed with 0.15 M phosphate-buffered saline (PBS), pH 7.2, and then incubated with RaMIg, 0.5–1.0 mg/ml, for two h at 4°C. Subsequently the beads were washed three times, incubated with RPMI-1640-10% FCS for 1 h in the cold, and loaded onto the column.

Lymphocytes to be separated were then introduced on top of the beads. Usually, the cells were at a concentration of 5×10^7 cells/ml and a maximum of 5×10^6 cells were used for every milliliter of beads. Separation was carried out at room temperature, but the medium was 4°C. Unattached cells were collected at a flow rate of 40–45 s/cm until the eluate became clear. The column was then washed with 15 ml of medium. Cells retained by the glass beads were freed into the supernate by swirling the beads in a beaker with RPMI-1640-5% FCS. Total yield from this procedure ranged from 75 to 93%.

Detection of sIg. 0.05 ml of fluorescein-conjugated RaMIg (N. L. Cappel Laboratories Inc.), centrifuged at 100,000 *g* for 1 h immediately before use, and diluted 1:10 was added to 1×10^6 lymphocytes in 0.1 ml of medium with 0.02 M sodium azide. This mixture was incubated for 15 min in a humidified 37°C incubator. Subsequently, unabsorbed antibody was removed by centrifuging the cells through 3 ml of neat FCS at 900 rpm for 5 min in the cold. After the supernate was poured off, the cell pellet was resuspended in 1 ml of PBS-50% FCS. Smears made from 0.25-ml aliquots of this suspension on a cytocentrifuge (Shandon Elliot Co., Sewickley, Pa.) were air-dried, fixed for 1 min in 10% formalin in ethanol, rehydrated for 5 min in PBS, and dried and mounted with 10% glycerol in PBS. Microscopy was performed with a Zeiss microscope (Carl Zeiss, Inc., N.Y.) with Ploem optics. Smears were read alternately under phase and UV illumination for the percentage of fluorescent-positive lymphocytes. The criterion for positivity was uniform punctate surface staining. Only cells which appeared to be lymphocytes by morphologic criteria were counted. A minimum of 200 lymphocytes was examined per preparation. 40–50% of splenocytes were stained by this procedure but no staining of thymocytes could be detected.

Removal of Phagocytic Cells

INCUBATION WITH CARBONYL IRON (FeC). A procedure modified from Erb and Feidman (21) and Sjöberg et al. (22) was employed. 10 mg/ml of FeC was added to a beaker containing lymphocytes at 20×10^6 cells/ml of RPMI 1640-10% FCS. The mixture was incubated at 37°C for 1 h during which the cells were agitated gently every 5 min. Subsequently, cells with phagocytized or adhering iron particles were retained using a strong magnetic field, whereas cells in the supernate were collected with a Pasteur pipet. This procedure was repeated three times. After two washes, the cells were counted and the efficiency of depletion estimated. The average recovery of nonphagocytic cells was ≅ 85%.

SEPHADEX G-10 COLUMNS (23). Sephadex G-10 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) was swollen in three to four times the estimated bed volume of normal saline. After swelling, the Sephadex G-10 was washed three times in the same volume, then resuspended in 1.3 times the bed volume of saline. The slurry was aliquoted in 35- to 40-ml amounts in glass tubes. Autoclaved glass beads (prepared as described under separation of Ig^+ cells) were added to a 50 ml syringe barrel (Monoject, Sherwood Medical Industries Inc., St. Louis, Mo.) to the 5-ml mark followed by Sephadex G-10 to the 35-ml mark. The column was washed with 15 ml of RPMI-1640-10% FCS warmed to 37°C. Then 5–8 ml of cells at 50×10^6/ml were introduced and nonadherent cells collected immediately until the filtrate was clear. About 80–90% of the lymphocytes introduced were recovered as nonadherent cells.

Detection of Phagocytic Cells

LATEX INGESTION. 10% latex particles (Dow Diagnostics, Indianapolis, Ind.) were diluted to 0.2% with RPMI-1640. 2×10^6 cells in 1 ml of the latex solution were incubated for 20 min at 37°C and smears made on the cytocentrifuge. The percentage of cells with ingested latex particles was determined microscopically after smears were stained with Wright's stain.

INDIA INK INCUBATION. 5×10^6 cells in 1 ml of RPMI-1640 with 5% FCS were placed in a 35-mm petri dish. 1–2 drops of sterile Higgins India ink diluted 1:10 with saline were added. After overnight incubation, the number of cells containing ink particles were counted.

ESTERASE STAINING. The method developed by Yam et al. (24) and Barka and Anderson (25) was used.

Results

Effect of Mitomycin-C Treatment on the Synergistic Activity of Nylon Wool-Adherent Spleen Cells. Previous work demonstrated that the lymphocytes responsive to LPF are T

TABLE I

Effect of Mitomycin-C on the Proliferative Response of Nylon Wool-Separated Spleen Cells

Spleen cells × 10^6 per culture	Mitogens			
	0.5 μg LPF	0.18 μl PHA-P	1.25 μg LPS	None
Unseparated 0.50	88.4 ± 3.7*	53.7 ± 2.9	58.1 ± 3.1	7.1 ± 0.4
NA 0.50	13.5 ± 1.4	—‡	—	0.9 ± 0.1
A 0.50	7.2 ± 1.5	—	—	1.5 ± 0.1
NA 0.25	11.4 ± 0.2	24.2 ± 3.1	3.2 ± 0.7	0.1 ± 0
A 0.25	3.7 ± 1.6	0.5 ± 0	51.8 ± 1.8	0.3 ± 0
NA_m 0.25	0.1 ± 0	0.2 ± 0	0.2 ± 0	0.1 ± 0
A_m 0.25	0.2 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0
NA 0.25 + A 0.25	92.2 ± 10.3	—	—	0.8 ± 0.2
NA 0.25 + A_m 0.25	72.1 ± 6.8	—	—	0.3 ± 0.1
NA_m 0.25 + A 0.25	10.4 ± 3.4	—	—	0.2 ± 0
NA_m 0.25 + A_m 0.25	2.1 ± 0.1	—	—	0.1 ± 0

Proliferative response of nylon wool-nonadherent spleen cells (NA) in the presence of nylon wool-adherent spleen cells (A) was assayed with or without pretreatment of either fraction with mitomycin-C. Individual cultures contained the indicated number of cells in 0.25 ml whereas cocultures were at 0.5 × 10^6 cells in 0.25 ml. NA_m and A_m denote the NA and A fractions after incubation with mitomycin-C.

* Mean cpm × 10^{-3} ± SD of triplicate cultures at 72 h.

‡ Not determined.

cells bearing Thy-1 antigen on their surfaces (5) but that T cell-enriched nonadherent cells from nylon wool columns did not respond unless cocultured with adherent cells. Because the adherent cell population recovered from nylon wool columns contained small numbers of T cells, it was possible that the proliferation observed in cocultures of nonadherent and adherent splenocytes was a result of the proliferation of the adherent T cells, whereas the T-cell-enriched, nonadherent fraction served as the accessory cell population. Therefore, coculture experiments were performed in which the proliferation of the different cell populations was inhibited by mitomycin-C, an inhibitor of DNA synthesis. As seen in Table I, mitomycin-C abolished the proliferative response of nonadherent cells to the T-cell mitogen PHA-P and of adherent cells to the B-cell mitogen LPS. In cocultures of untreated nonadherent and mitomycin-C-treated adherent cells, the response of LPF was not significantly different from that in cocultures of untreated nonadherent and untreated adherent cells. Similar results were obtained when nonadherent and adherent cells were mixed in ratios of 1:3 and 3:1. In contrast, mitomycin-C treatment of nonadherent cells abolished the proliferative response to LPF in cocultures. Thus, it was confirmed that it was the nonadherent cells, and not the adherent cells, which were stimulated by LPF and that the adherent cell fraction contained the accessory helper cells.

Synergistic Effect of Nylon Wool-Adherent Cell Fractions from Bovine Serum Albumin (BSA) Density Gradients. In studying the proliferative response to PHA by a mixture of lymph node cells and syngeneic thymocytes, Piquet et al. (26) attributed the augmented response of the mixture to a nonspecific dilution of lymph node blasts. To determine whether this was also the case with LPF stimulation, and to provide information on the density of the accessory cells, adherent spleen cells were fractionated on a discontinuous BSA gradient (Table II). The synergizing effect was associated predominately with cells with light densities, i.e. fraction A at the 10–23% interface and fraction B at the 23–26% interface. Fractions C (26–29%) and D (29–32%)

TABLE II

Synergistic Effect of Nylon Wool-Adherent Cell Fractions from BSA Density Gradients

Spleen cells × 10^6 per culture	Mitogens		
	0.5 μg LPF	0.5 μg Con A	1.25 μg LPS
Unseparated 0.50	123.3 ± 3.6*	151.6 ± 10.7	64.1 ± 2.2
NA 0.25	19.2 ± 4.4	108.6 ± 8.1	0.3 ± 0.2
A 0.25	0.8 ± 0.1	1.6 ± 0.7	18.3 ± 0.6
A_A 0.25	0.2 ± 0.1	0.8 ± 0.1	39.5 ± 4.7
A_B 0.25	0.1 ± 0.1	0.4 ± 0.2	16.3 ± 0.1
A_C 0.25	0	0	1.0 ± 0.2
A_D 0.25	0	0	0.3 ± 0.1
NA 0.25 + A 0.25	102.9 ± 9.8	112.3 ± 3.1	28.2 ± 6.4
NA 0.25 + A_A 0.25	101.3 ± 8.0	144.2 ± 4.4	30.1 ± 3.9
NA 0.25 + A_B 0.25	97.1 ± 9.4	165.9 ± 4.6	12.9 ± 3.9
NA 0.25 + A_C 0.25	28.2 ± 6.4	131.1 ± 27.2	4.3 ± 3.8
NA 0.25 + A_D 0.25	15.4 ± 2.9	126.8 ± 3.6	0.8 ± 0

Nylon wool-adherent spleen cells were fractionated on discontinuous BSA gradients as described in Materials and Methods, A_A, A_B, A_C, and A_D denote cell fractions collected from interfaces of 10–23%, 23–26%, 26–29%, 29–32% BSA, respectively. 0.25 × 10^6 cells from each fraction were either cultured alone or cocultured with 0.25 × 10^6 nylon wool-NA spleen cells in 0.25 ml.

* Net mean cpm × 10^{-3} ± SD of triplicate cultures at 72 h.

exhibited little or no enhancing properties. The pellet contained few cells and these did not synergize (data not shown). Hence, the synergy observed was not simply a dilution effect but was clearly associated with a distinct subpopulation(s) of adherent cells. It is of note that fractions A and B, which contain the bulk of the accessory cells, also contain the majority of LPS-responsive cells. Because total recovery from the four BSA interfaces was ≅ 90%, the presence of helper cells in fractions A and B was not a result of excess cell loss leading to false enrichment of accessory cells in these fractions.

Effect of Thy-1.2 Antiserum on Synergizing Properties of Adherent Spleen Cells. Adherent spleen cells recovered from nylon wool columns are only enriched in B cells and may be contaminated with T lymphocytes which could serve as helper cells for the responding T cells in the nonadherent fraction. These residual T cells may also account for the background proliferation of the adherent population. Such T-T interactions have been previously observed in mitogenic responses to Con A (27–30). Adherent spleen cells were therefore treated with Thy-1.2 antiserum and complement to remove the residual T cells. As seen in Table III, treated adherent cells not only lost their residual response to PHA-P and Con A, the slight LPF response was also abolished. A cell mixture containing equal numbers of nonadherent cells and Thy-1.2 antiserum-treated adherent cells gave a similar response as a mixture of nonadherent cells and untreated adherent cells. Thus, the cells responsible for the enhancing effect are not sensitive to treatment with Thy-1.2 antiserum. Because the responding cells are Thy-1^+ (5), the above finding also indicates that the proliferating cells in a mixture of nonadherent and adherent splenocytes are of a nonadherent origin.

Effect of Macrophage Depletion on Synergy. Several workers have suggested that macrophages can act as accessory cells in the stimulation of lymphocytes by T-cell mitogens (31–32). To explore the effect of the removal of macrophages on LPF

TABLE III

Effect of Thy-1.2 Antiserum on Synergizing Properties of Nylon Wool-Adherent Spleen Cells

Spleen cells	Mitogens		
	LPF 0.5 μg	PHA-P 0.18 μl	Con A 0.5 μg
Unseparated	190.8 ± 4.6*	71.8 ± 5.6	222.8 ± 10.0
NA	52.2 ± 5.5	108.0 ± 3.2	264.5 ± 9.3
A (untreated)	35.4 ± 6.1	2.5 ± 1.6	63.7 ± 23.7
A (treated)	0	0	0
NA + A (untreated)‡	148.2 ± 8.2	ND§	ND
NA + A (treated)‡	150.1 ± 10.4	ND	ND

* Net mean cpm × 10^{-3} ± SD of triplicate cultures containing 0.5 × 10^6 cells/0.25 ml at 72 h.

‡ Mixtures contain equal numbers of NA and A cells.

§ ND, not determined.

TABLE IV

Effect of Macrophage Removal by Carbonyl Iron on the Proliferative Response of Spleen Cells to LPF

Spleen cells	Mitogens		
	None	LPF 0.5 μg	Con A 0.5 μg
Untreated	7.7 ± 1.2*	164.5 ± 12.3	242.5 ± 37.0
Treated‡	0.4 ± 0.1	148.9 ± 9.6	202.3 ± 16.9

* Mean cpm × 10^{-3} ± SD at 72 h of triplicate cultures containing 0.5 × 10^6 cells/0.25 ml.

‡ Treated spleen cells were incubated with FeC, 100 mg/200 × 10^6 cells, for 1 h at 37°C.

TABLE V

Effect of G-10 Column Separation of CBA Spleen Cells on Synergy

Spleen cells	Untreated	Treated*
Unseparated	130.1 ± 5.6‡	120.4 ± 4.1
Nonadherent (NA)	23.7 ± 0.6	20.7 ± 1.2
Adherent (A)	2.0 ± 0.3	1.5 ± 0.2
NA + A (1:1)	125.1 ± 5.7	142.9 ± 6.4

* Cells passed through a column of Sephadex G-10 as described in methods.

‡ Net mean cpm ± SD from triplicate cultures containing 0.5 × 10^6 cells/0.25 ml harvested at 72 h.

mitogenesis, spleen cells were depleted of macrophages by incubation with carbonyl iron (21, 22). The macrophage-depleted spleen cells gave a proliferative response to LPF and Con A equivalent to that of untreated spleen cells (Table IV). Further experiments entailed the removal of macrophages from both whole spleen cells and adherent cells by passage through a Sephadex G-10 column (23). Treated spleen cells responded to LPF as well as the untreated cells (Table V). Moreover, treated adherent cells still retained their amplifying effect. Nor was the residual LPF response in the nonadherent fraction abrogated by column passage. Efficiency of macrophage depletion was monitored by esterase staining and phagocytosis of latex or India ink particles. The tests demonstrated that <1% of the treated cells were macrophages.

TABLE VI

Synergistic Effect of Spleen Cells Separated on Anti-Ig Columns

Spleen cells × 10^6/culture	cpm × 10^{-3} ± SD	Percent Ig^+ Cells*
Unseparated 0.50	122.2 ± 9.2‡	48
NA 0.25	6.3 ± 0.5	3
A 0.25	2.5 ± 0.6	72
A_F 0.25	0.5 ± 0.2	7
A_S 0.25	0	89
NA 0.25 + A 0.25	93.4 ± 10.8	
NA 0.25 + A_F 0.25	9.7 ± 4.0	
NA 0.25 + A_S 0.25	88.2 ± 7.1	
NA 0.15	5.3 ± 2.3	
A 0.35	2.8 ± 0.8	
A_F 0.35	0.5 ± 0.1	
A_S 0.35	0.3 ± 0.3	
NA 0.15 + A 0.35	65.5 ± 2.9	
NA 0.15 + A_F 0.35	1.6 ± 0.9	
NA 0.15 + A_S 0.35	59.2 ± 8.3	

Nylon wool-A spleen cells were passed through a column of glass beads coated first with mouse Ig, then rabbit anti-mouse Ig. A_F represents the cells in the effluent whereas A_S denotes the cell fraction retained by the glass beads. Indicated numbers of A, A_F, or A_S were either cultured alone or in the presence of nylon wool-NA spleen cells.

* As detected by staining with fluorescein-conjugated rabbit anti-mouse Ig serum.

‡ Net mean cpm × 10^{-3} ± SD from triplicate LPF (0.5 μg) cultures containing the indicated number of cells in 0.25 ml at 72 h.

Thus, these results, together with previous findings (5), indicate that the synergizing effect is not associated with typical macrophages.

Synergistic Effect of Spleen Cells Passed through Anti-Ig Columns. Dyminski and Smith (34) observed that the mixed lymphocyte reaction (MLR) between low density thymus cells required the contribution of an sIg^+ cell. To explore the possibility of an Ig-bearing helper cell in our system, lymphocytes were passed through an anti-Ig column which retained cells bearing sIg and/or FcR (35). As detected by immunofluorescent staining, adherent spleen cells, after column-passage, contained only 7% sIg^+ cells, whereas 89% of the cells retained on the column were Ig^+. Only cells retained on the column possessed synergizing properties (Table VI) when tested with nonadherent cells. The possibility of a sticky synergizing cell nonspecifically retained by the column is unlikely because the helper cells were not retained by a column of glass beads coated first with mouse Ig, and then normal rabbit serum.

Effect of Anti-Immunoglobulin Sera on Synergy. Results from the studies using anti-Ig columns indicated that the helper cells are sIg^+ and/or possess receptors for the Fc portion of immunoglobulin (FcR^+). To further characterize this cell population, both nonadherent and adherent lymphocytes were treated with antisera against mouse immunoglobulins and complement before admixture. The degree of sIg^+-cell depletion was monitored by immunofluorescence. Greater than 95% of the sIg^+ cells in both fractions were routinely lysed by such treatment. Moreover, the LPS responses of these fractions were abolished but their Con A responses remained unchanged or slightly increased (results not shown). As seen in Fig. 1, various doses of untreated

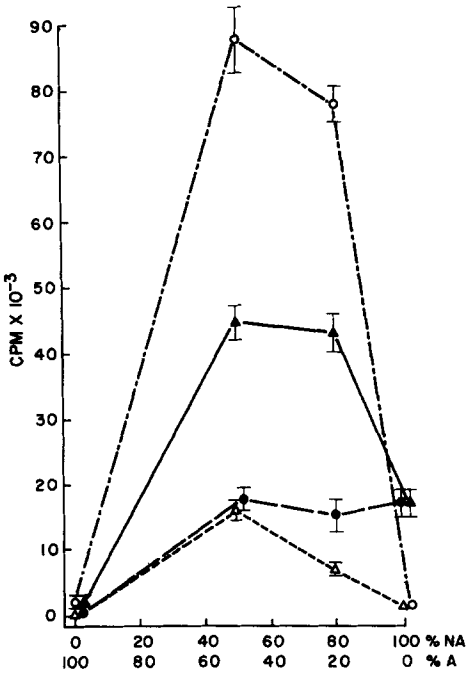

FIG. 1. Effect of cytolysis with anti-Ig serum on synergy. Both nylon wool-nonadherent (NA) and adherent (A) splenocytes were depleted of sIg^+ cells by cytolysis as described in methods. The proliferative response of cell mixtures containing the indicated percentages of treated NA and untreated A (○), untreated NA and untreated A (▲), untreated NA and treated A (●), and treated NA and treated A (△), splenocytes were assayed by ^3H-thymidine incorporation at 72 h. All cultures contained 0.5×10^6 cells in 0.25 ml.

adherent cells were synergistic for treated or untreated nonadherent cells, whereas anti-Ig treated adherent cells were not synergistic. The background proliferation of nonadherent cells when cultured alone was also abolished after the cytolysis of sIg^+ cells. Because the dead cells were removed by centrifugation through a Ficoll-Conray gradient, it is reasonable to assume that the elimination of the helper function of adherent cells after cytolysis was not a result of toxic substances released from these dead lymphocytes but a depletion of the cell population responsible for synergy. Because the presence of sIg, but not FcR, on cell surfaces renders the cells sensitive to lysis by anti-mouse immunoglobulins and complement, it is apparent that the helper population is sIg^+. Furthermore, treated nonadherent cells were more responsive than their untreated counterparts in the presence of adherent cells. This is probably due to a relative enrichment of responding T cells in the treated nonadherent cells. No effect on LPF response was observed when normal rabbit serum was used instead of anti-Ig serum.

Synergistic Effect of sIg^+ on sIg^- Splenocytes Fractionated on a FACS. The loss of helper function from the adherent cells after removal of sIg^+ lymphocytes strongly suggested that helper cells bear immunoglobulins on their surfaces. If this hypothesis is indeed correct, then a pure population of sIg^+ cells would be synergistic whereas their sIg^- counterparts would not. The sIg^+ or sIg^- fractions obtained by passage through an anti-Ig column are enriched for these cells but they are by no means pure populations. This prompted us to perform fractionations on a FACS where lymphocytes stained

with fluorescein-conjugated RaMIg are separated into bright and dull populations according to the intensity of fluorescence.

There were three differences in the design of the FACS experiments as compared to the former studies. Firstly, BAB/14 mice were used instead of CBA mice. Both strains are responsive to LPF although the response of spleen cells of the former is usually lower. Nevertheless, no variation was noted in the synergistic phenomenon when BAB/14 mice were used. The second difference lies in the final cell densities of the mixed cultures. In the previous studies, nonadherent cells were mixed with adherent cells in various ratios whereas the final cell number per mixed culture remained constant. Here, 4×10^5 nonadherent cells were mixed with $0\text{–}1 \times 10^5$ helper cells in each culture. Because this number of nonadherent cells represents an excess of responder cells (M.-K. Ho, unpublished observation), the limiting factor for proliferation is the number of helper cells added. It is obvious that the final cell densities increased with an increase of helper cells in culture; but an increase in total cell number from 4×10^5 to 5×10^5 cells per culture should not markedly affect the proliferative response. Moreover, as long as both sIg^+ and sIg^- cells are added to the responder lymphocytes in the same manner, then it is valid to compare the two fractions with respect to their synergistic ability. Lastly, whole spleen cells, instead of nylon wool-adherent cells, were employed as a source of helper cells to minimize the manipulations, and to preserve viability of the cells.

In Fig. 2, the helper effect of sIg^+ splenocytes is compared to that of sIg^- cells. The control source of helper cells were spleen cells stained with antiserum, but not passed through the FACS. It was found that 1×10^5 (the maximum number used) of stained but unseparated spleen cells or sIg^- cells yielded $\cong$ 10,000 and 4,800 cpm, respectively, when cultured alone with LPF, whereas minimal incorporation (800 cpm) was observed with 1×10^5 sIg^+ cells. It is clear that the LPF response of nonadherent cells increased when increasing numbers of stained unseparated spleen cells or separated sIg^+ cells were added, but sIg^- cells had no effect. Furthermore, reminiscent of the synergy between nylon wool-adherent and nylon wool-nonadherent cells, optimal LPF response was achieved by a mixed culture containing sIg^+ and sIg^- splenocytes, whereas both populations responded minimally when cultured alone.

Synergistic Effect of CR^+ and CR^-. Cells bearing CR are of bone marrow origin (36). However, not all bone marrow-derived or B cells are CR^+ (36, 37). To determine whether the CR-bearing subpopulation of B lymphocytes is responsible for synergy, the helper function of CR^+ and CR^- nylon wool-adherent spleen cells separated by EAC rosettes were assayed. As seen from Fig. 3, the CR^+ population exhibited similar synergistic activity as the unseparated cells. However, significant helper function was also detected in the CR^- fraction. It is apparent that the helper lymphocytes reside in both the CR^+ and CR^- fractions of nylon wool-adherent splenocytes, though the former is more synergistic.

Discussion

Previous studies showed that LPF was mitogenic for T lymphocytes from adult mouse spleen, peripheral lymph node, and cortisone-treated thymus, but not normal thymus or bone marrow (4). Although nylon wool-nonadherent splenocytes responded well to the T-cell mitogens, Con A and PHA, and nylon wool-adherent spleen cells to the B-cell mitogen, LPS, neither cell type was stimulated optimally by LPF (5).

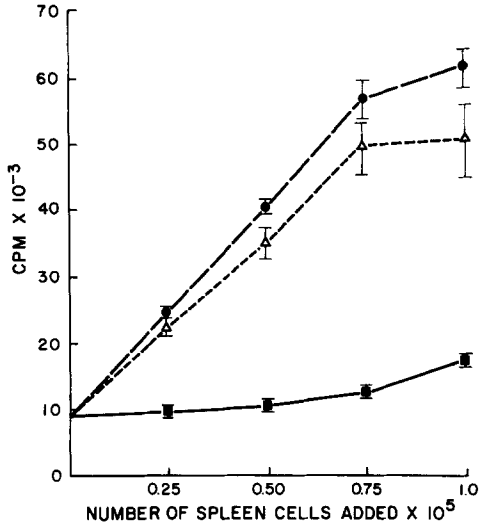

FIG. 2. Synergistic effect of sIg^+ and sIg^- spleen cells separated on a FACS. Spleen cells were stained for surface immunoglobulins and separated into sIg^+ and sIg^- fractions according to their intensity of fluorescence. Indicated number of sIg^+ (●) or sIg^- (■) cells were added to 0.4×10^6 NA cells per culture and their synergistic effect compared to control spleen cells which were stained but not fractionated on the FACS (△). All cultures were harvested at 72 h.

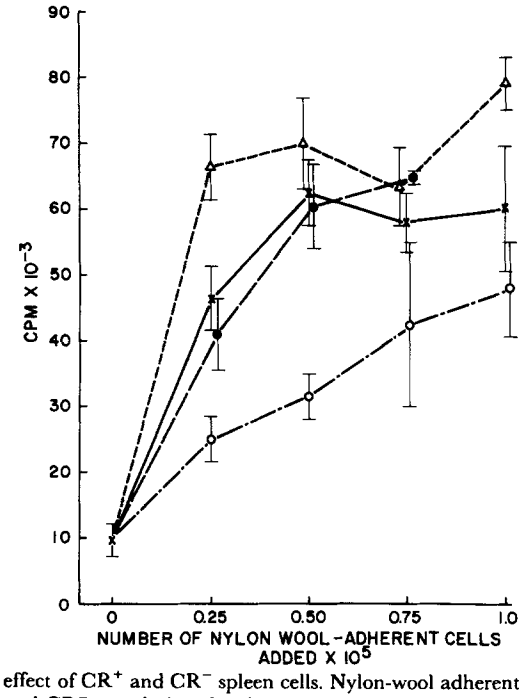

FIG. 3. Synergistic effect of CR^+ and CR^- spleen cells. Nylon-wool adherent spleen cells (A) were separated into CR^+ and CR^- populations by sheep erythrocytes sensitized with anti-sheep erythrocyte antibody and mouse C3 rosettes. Indicated numbers of unseparated (×), CR^+ (△), CR^- (○), or a mixture of CR^+ and CR^- (1:1) (●) adherent cells were added to 0.4×10^6 nylon wool-NA spleen cells in 0.25 ml. When cultured alone at 1×10^5 cells/culture, the proliferative response of unseparated, CR^+, and CR^- adherent cells were 200, 100, and 3,000 cpm, respectively. The ^{3}H-thymidine incorporation of 5×10^5 whole spleen cells when cultured alone was 92,500 cpm. All cultures were harvested at 72 h.

However, optimal LPF mitogenic response was restored when both cell types were cultured together. This synergistic phenomenon is not restricted to lymphocytes from CBA mice because similar observations were made using other mouse strains such as BAB/14, BALB/c, DBA/2, and B10D2/nSn (M.-K. Ho, unpublished observation).

There are several interpretations for this phenomenon. First, the small number of contaminating T cells adherent to nylon wool might proliferate in the presence of nonadherent lymphocytes. This possibility can be ruled out because mitomycin-C treatment of the nonadherent cells, but not the adherent cells, abolished synergy (Table I). Therefore, the proliferating T lymphocytes were clearly shown to be in the nonadherent fraction.

Another possible explanation is that the synergistic effect observed is simply a result of different cell densities in cultures containing only one cell type, compared to those with mixed cell types. This is unlikely because only the light-density fractions of adherent spleen cells were synergistic. Moreover, increasing the cell densities of the individual cell types (namely, nonadherent or adherent splenocytes) did not increase their response to LPF (Table I).

The most likely interpretation is as follows: T lymphocytes in the nylon wool-nonadherent fraction respond to LPF only in the presence of one or more distinct subpopulation(s) of nylon wool-adherent cells. These accessory cells possess relatively light buoyant density and still exert their effect after pretreatment with mitomycin-C.

Because a number of cell types, including T cells, macrophages, B cells, null cells, and precursor cells, were present in the nylon-adherent fraction, the nature of the helper cell population was unclear. This prompted us to perform studies on the surface markers and adherence properties of these cells.

T-T-cell interactions have been implicated in the proliferation of lymphocytes to Con A (27–30) and PHA (26). However, the synergy between subpopulations of T cells in LPF-induced blastogenesis is improbable. First, the synergistic, lighter density fractions of spleen cells separated on BSA gradients are virtually devoid of Thy-1^+ lymphocytes (38). Second, cytolysis of Thy-1 bearing cells from the adherent fractions (Table III) did not abolish their synergistic effect. Moreover, we have shown that even though fetal liver cells are not synergistic, synergistic activity can be detected in spleens of adult thymectomized mice, lethally X-irradiated and reconstituted with Thy-1 antiserum-treated fetal liver cells. Therefore, the development of helper cells from their fetal liver precursor cells is thymus-independent.[2]

Further experiments on the properties of the helper cells indicated that typical macrophages were not responsible for the helper function because neither treatment with FeC nor passage through Sephadex G-10 columns abrogated the synergistic effect of splenocytes. This finding is consistent with previous experiments by Kong and Morse (5) in which the optimal LPF response of a culture of nylon wool-nonadherent spleen cells was not restored by addition of either up to 20% peritoneal exudate cells, or varying quantities (10–50 μM) of 2-mercaptoethanol. Moreover, splenic macrophages were also not synergistic (M.-K. Ho, unpublished observations).

The observation that helper activity of nylon wool-adherent cells can be removed by (*a*) passage through anti-Ig columns and (*b*) lysis with RaMIg and complement,

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[2] Ho, M.-K., A. S. Kong, and S. I. Morse. The in vitro effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. IV. Synergistic and suppressive effect of unresponsive cells. Manuscript in preparation.

strongly suggests that the helper cells are sIg^+. Even though both sIg^+ and/or FcR^+ cells can be retained by anti-Ig columns, cells bearing only FcR, but not sIg, are unlikely to be susceptible to complement-mediated lysis.

Critical evidence pointing toward the presence of sIg on helper cells comes from the fact that sIg^+ spleen cells separated on the FACS are synergistic. Slight synergy can be detected in the sIg^- fraction when used at high concentrations but this can be accounted for by the presence of B cells bearing low densities of sIg. Because the staining reagents are freed of aggregates immediately before use, and FcR^+ cells do not bind monomeric Ig readily (39), the presence of sIg^- FcR^+ cells in the brightly stained fraction is highly unlikely. It appears that the helper cells are sIg^+ and therefore of B-cell lineage; moreover, bone marrow cells, but not thymocytes, also possess synergistic activity.

The detection of sIg on helper cells by no means implies that all sIg^+ cells are synergistic. It is conceivable that only one or more subpopulation(s) of B cells is responsible for the helper activity. Ontogenic studies[2] reveal that helper activity can be detected in the spleen only after 7 d of age, and it increases as the animal matures. In view of the fact that spleen cells bearing IgM, IgG_2, or IgA appear early in life (40–42), it is unlikely that these Ig molecules are the determining markers for helper activity. However, the possibility of an IgD^+-helper cell cannot be ruled out (43, 44). Experiments are now in progress to determine whether helper activity is associated with a specific immunoglobulin isotype by using antiserum to individual heavy chains. The relatively late ontogenic development of CR^+ cells (41, 45–57) also prompted us to explore the role of these cells in synergy. Thus far, helper activity appears to reside in both CR^+ and CR^- nylon wool-adherent spleen cells.

In addition to classification of isotypes, Ig-bearing cells can be subgrouped according to their densities of sIg. Scher et al. (48, 49) observed that the number of cells bearing high densities of sIg are high in neonatal mice. As the animals mature, the number of these cells decreases accompanied by a concomitant increase in cells bearing sIg of low to intermediate densities. A defect in the maturation process of B cells in CBA/N mice results in an abnormally high number of cells bearing high density sIg even in adults (48, 49). Accordingly, the number of cells with low to intermediate density of sIg is diminished. Preliminary results indicate that spleen cells from CBA/N mice are responsive to LPF. Moreover, significant helper activity can be detected in their nylon wool-adherent cells as well as in bone marrow cells. Therefore, it appears that helper activity is not exclusively associated with B-cell subpopulations bearing low to intermediate densities of sIg.

There is evidence from many types of experiments indicating the involvement of Ia antigens in cellular interactions (50, 51). It would be intriguing if Ia-bearing cells indeed play a role in synergy in the LPF system. In addition to Ia antigens, the involvement of other markers found on B cells such as FcR, antigens determined by the minor lymphocyte-stimulating locus (52, 53) and Ly-4 (54, 55) are currently under investigation.

Experiments are in progress to elucidate the events during synergy. Preliminary results suggest that soluble factors are probably not involved in the cooperation because neither culture supernates of unseparated nor of nylon wool-adherent splenocytes can substitute for the helper cells. However, the requirement of a short-lived mediator cannot be ruled out. The triggering of T lymphocytes in the presence of

LPF may entail two signals. The first signal being the mitogen itself whereas the second comes from the helper lymphocytes. This interpretation is based on experiments in which nylon wool-nonadherent cells were mixed with nylon wool-adherent cells and LPF at various times. It was found that the optimal LPF response at 72 h of culture could be achieved when adherent cells were added as late as 18 h after nonadherent cells were incubated with LPF. However, a delay in the addition of LPF to an admixture of nylon wool-nonadherent and nylon wool-adherent cells led to a delay in time of optimal proliferation.

Dyminski and Smith (34) observed that IgG could substitute for the requirement of helper B cells in the mixed lymphocyte reaction between immunocompetent mouse thymus cells. They suggested that FcR may be involved in cellular cooperation in their system. Whether Ig of various isotypes and their respective fragments can substitute for helper cells in LPF-induced proliferation awaits further investigation.

Because no B-cell-dependent T-cell mitogen has been reported previously, the requirement of a B-helper cell in LPF-induced proliferation of mouse T cells is a novel finding. In addition to defining a new functional role for B lymphocytes, studies in this simple in vitro system can provide insight into the dynamic interplay among various lymphocyte subpopulations and their mechanism of interaction. Moreover, because cytotoxic T lymphocytes are generated in the presence of LPF after 72 h of culture,[3] understanding the events leading to LPF-induced proliferation could also shed light on the generation of cytotoxicity.

Summary

The nature of the helper lymphocytes in lymphocytosis-promoting factor (LPF)-induced proliferation was explored. Removal of macrophages from adherent splenocytes by either carbonyl-iron incubation or passage through Sephadex G-10 columns did not affect their synergistic function. Nor did cytolysis with Thy-1.2 antiserum and complement. The helper cells were found to be surface immunoglobulin-positive (sIg^+) because they are retained by anti-Ig columns, susceptible to lysis by rabbit anti-mouse immunoglobulin and complement, and occurred in the sIg^+ fractions of splenocytes after separation on the fluorescence-activated cell sorter. Further delineation of the surface markers on helper cells showed that complement receptors are not the determining marker for synergistic function. The requirement for B-helper cells in the stimulation of T lymphocytes by LPF is unique for a mouse T-cell mitogen.

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