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Mitogen-Induced Amplification of Blastogenesis in Lipopolysaccharide-Precultured Lymphocytes

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Human peripheral blood lymphocytes were cultured in vitro with lipopolysaccharide (LPS) for 48 h. After washing, stimulation with a concanavalin A (ConA) or pokeweed mitogen (PWM) resulted in a synergistic blastogenic response that was greater than the sum of the independently stimulated control cultures. Addition of fresh autologous lymphocytes after LPS preculture produced an additional increment of synergy. The nature of the responding and helping effects was determined by coculturing irradiated or nonirradiated lymphocyte suspensions that had been precultured with either LPS or ConA/PWM. Such studies indicated that amplification was the result of a mitogen-activated helper activity, which facilitated the blastogenic response to LPS. Experiments with lymphocytes resolved into T- and B-cell-enriched fractions indicated that the LPS-responsive cells were of the B type and that the help was provided by a mitogen-activated T-cell population. These studies indicated that LPS can induce human B-cell blastogenesis; however, a helper function must be provided by a T-cell subpopulation. This helper activity is inducible by pretreatment of the T cells with plant lectins.

Lipopolysaccharide (LPS) obtained from gram-negative bacteria has been shown to be a potent stimulant of blastogenesis and immunoglobulin synthesis in both spleen and peripheral blood lymphocytes of mice (2, 8, 9, 18). However, in humans, LPS is a poor stimulant of blastogenesis of spleen, lymph node, tonsil, and bone marrow lymphocytes, and is virtually nonstimulatory to peripheral blood lymphocytes under similar cultural conditions (21; R. R. Rich and J. M. Chandler, Clin. Res. 24:S335, 1976). Human lymphocytes, however, will respond blastogenically to LPS stimulation after lengthy incubations (7 to 9 days) (17). We have previously reported that mitogenic stimulation of human lymphocytes with concanavalin A (ConA) or pokeweed mitogen (PWM) is capable of generating a helper activity (15). Such activity is capable of augmenting the blastogenic response of lymphocytes which have been stimulated with ultrasonic fluids of *Actinomyces viscosus*.

Previous reports (23) indicated that mixtures of mitogens and LPS were synergistic; i.e., the blastogenic response to the mixtures was greater than the sum total response of independently cultured stimulants. This report describes current studies using a preculture system, whereby distinct lymphocyte subfractions were cultured with either LPS or lectin. These studies indicate that a helper function can be activated in peripheral blood lymphocytes which can facilitate the B-lymphocyte response to LPS.

MATERIALS AND METHODS

Isolation of mononuclear leukocytes from human peripheral blood. Mononuclear leukocytes were isolated from heparinized venous blood drawn from 12 healthy volunteers between the ages of 21 and 40. A leukocyte-rich buffy-coat layer was obtained from whole blood by centrifugation at 350 × *g* for 30 min. The buffy coat was diluted in sterile phosphate-buffered saline, layered onto Ficoll-Hypaque (3) (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, N.J.), and centrifuged at 500 × *g* for 30 min at 23°C. The cells banded at the interface were aspirated, washed three times in phosphate-buffered saline, and suspended to a final concentration of 2×10^6 cells/ml in RPMI 1640 culture medium (GIBCO Laboratories, Grand Island, N.Y.), which was supplemented with 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 2 mM glutamine, and penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively).

T- and B-lymphocyte isolation. Mononuclear leukocyte suspensions were depleted of monocytes by the Sephadex G-10 column method (1, 12). Passage through such columns produced lymphocyte suspensions with less than 1% monocyte contamination as determined by latex particle uptake (20) and nonspecific esterase staining (14).

T cells were prepared from these suspensions by rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (SRBCs) (13). Human T cells binding SRBCs were separated from nonrosetted

cells by centrifugation through a Ficoll-Hypaque gradient as previously described. The T cells that pelleted at the bottom of the gradient were isolated by lysing the SRBCs with 0.85% NH_4Cl. Such T-cell preparations were typically free of B-cell and monocyte contamination (>99% T cell). The nonrosetting cell fraction at the gradient interface was typically >90% B cells. B lymphocytes were identified by rosetting with anti-human immunoglobulin-coupled polyacrylamide beads and by lack of nonspecific esterase staining (14).

LPS preparations and mitogens. LPS from *Escherichia coli* O127:B8 and *Serratia marcescens* (Difco Laboratories, Detroit, Mich.) was further purified by sedimentation in an ultracentrifuge at 100,000 × *g* for 4 h (19). LPS from *Bacteroides melaninogenicus* subsp. *intermedius*, supplied by Salam A. Syed (University of Michigan), was extracted by the phenol-water method of Westphal (26) and repurified by the method of Nowotny et al. (19). ConA was obtained from Pharmacia Fine Chemicals. PWM was obtained from GIBCO.

Preincubation of leukocytes with LPS and mitogens. Mononuclear leukocyte suspensions (2×10^6 cells/ml of RPMI 1640) in sterile polypropylene culture tubes (12 by 75 mm) with snap-on caps were cultured with LPS (1 to 1,000 μg/ml), PWM (1:10 to 1:1,000 dilution), or ConA (5 to 50 μg/ml). Control cultures were prepared without stimulants. After incubation for 16 to 48 h at 37°C in a humidified atmosphere containing 5% CO_2, the cells were washed and suspended in RPMI 1640 cultured medium. Viability was assessed by trypan blue dye exclusion and was found to be in excess of 90%. Total cell recoveries were 60 to 80% for all cell categories at 48 h.

Evaluation of modulating activity. Fresh autologous lymphocytes were obtained after the 48-h (stage I) incubation period. Fifty thousand cells were added to flat-bottom microtiter plate wells containing either 50,000 LPS-preincubated cells or 50,000 control cells. Mitogens were added to cultures (ConA, 0.1 to 100 μg/ml; PWM, 1:10 to 1:1,000 dilution) in triplicate wells. Four major culture categories were prepared: (i) control preincubated cells plus fresh cells (no stimulants); (ii) control preincubated (48 h) cells plus fresh cells and mitogen; (iii) LPS-preincubated (48 h) cells plus fresh cells; and (iv) LPS-preincubated (48 h) cells plus fresh cells and mitogen. In addition, all cultures contained a final concentration of 10% autologous plasma. The cell mixtures were incubated for an additional 3 days at 37°C. Mitomycin C (50 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) treatment of various cell categories was used to inhibit deoxyribonucleic acid (DNA) synthetic activity. Precultured cell categories were treated with mitomycin C for 30 min at 37°C and then washed three times with RPMI 1640. The second culture period will be referred to as stage II throughout this report.

Six hours before termination of stage II, 2 μCi of [*methyl*-^{3}H]thymidine (Amersham/Searle, Arlington Heights, Ill. TRA 120, 5 Ci/mmol) was added to each well. All cultures were harvested with a multiple automated sample harvester (MASH II, Microbiological Associates, Walkersville, Md.) onto glass fiber filters. The filters were dried and placed in vials (Bio-Vial, Beckman Instruments, Inc., Fullerton, Calif.), which were filled with 3 ml of scintillation cocktail (OCS, Amersham/Searle) and counted in a Packard model 3320 liquid scintillation spectrometer.

RESULTS

Effect of LPS pretreatment and subsequent mitogen stimulation on lymphocyte reactivity. In preliminary experiments, lymphocytes were precultured with *B. melaninogenicus* LPS at a concentration of 1,000 μg/ml for 48 h (stage I). After washing, half of the preincubated cells were treated with mitomycin C to determine whether stage I lymphocytes were capable of influencing the mitogen response of stage II cells. The influence of LPS pretreatment and subsequent ConA (Table 1) or PWM (Table 2) stimulation in stage II cultures was assessed. ConA stimulation of stage II cultures containing LPS-pretreated (stage I) cells resulted in blastogenic responses ranging from 1.47- to 3.7-fold greater than cultures containing control (stage I) cells. Mitomycin C treatment of the stage I cells abrogated the increased responses. Use of PWM in place of ConA yielded similar results.

TABLE 1. *Amplification of ConA stimulation of human lymphocyte blastogenesis after LPS pretreatment*[a]

Subject	LPS pretreatment	[^{3}H]thymidine incorporation (cpm)[b]			
		Without mitomycin C		With mitomycin C[c]	
		cpm ± SEM[d]	E/C[e]	cpm ± SEM	E/C
1	−	32,537 ± 1,667		36,187 ± 1,668	
	+	76,494 ± 5,595	2.35	35,024 ± 1,082	0.97
2	−	59,732 ± 4,392		38,665 ± 822	
	+	119,999 ± 7,553	2.01	28,926 ± 463	0.74
3	−	16,893 ± 725		6,565 ± 342	
	+	24,842 ± 259	1.47	6,511 ± 256	0.99
4	−	16,580 ± 2,177		11,673 ± 396	
	+	32,704 ± 1,189	1.97	11,420 ± 2,029	0.98
5	−	17,962 ± 530		11,291 ± 577	
	+	41,859 ± 2,766	2.33	10,956 ± 255	0.97
6	−	16,779 ± 2,714		13,369 ± 1,433	
	+	61,583 ± 518	3.67	10,883 ± 1,369	0.81
7	−	8,831 ± 1,347		10,093 ± 1,391	
	+	16,607 ± 712	1.88	9,518 ± 240	0.94
8	−	7,243 ± 246		8,589 ± 554	
	+	16,711 ± 474	2.31	8,063 ± 393	0.94

[a] Lymphocytes were precultured in the presence of *B. melaninogenicus* LPS (1,000 μg/ml) or medium alone for 48 h (stage I). After washing, fresh autologous cells and ConA (5 μg/ml) were added and cells were cultured an additional 3 days (stage II). Six hours before termination of culture, [*methyl*-^{3}H]thymidine was added to each culture.

[b] Expressed as Δcpm (cpm per 10^5 lymphocytes in mitogen-stimulated cultures minus cpm per 10^5 lymphocytes in unstimulated cultures).

[c] Stage I (precultured) cells treated with mitomycin C (50 μg/ml) for 30 min at 37°C before stage II culture.

[d] Standard error of the mean.

[e] Experimental/control (Δcpm of LPS-treated cultures/Δcpm of untreated cultures).

Effect of LPS preculture concentration. The optimal concentration of LPS required to generate a stage II amplified response was determined. LPS was tested at concentrations of between 10 and 1,000 μg/ml (Fig. 1). Concentrations of LPS over 1,000 μg/ml did not significantly enhance the effect (data not shown), whereas concentrations lower than 1,000 μg/ml showed little or no amplification.

Mitogen concentration dependency in stage II. ConA was tested between 0.5 and 50 μg/ml in 10-fold steps. At each concentration, a significant amplification of the LPS-mitogen response occurred when compared with the control cultures (Fig. 2). Similarly, PWM stimulation, tested between 1:1,000 and 1:10 of the stock solution, also resulted in significant amplification over control cultures (Fig. 3).

Comparison of different LPS batches. The general nature of the LPS preincubation effect was evaluated by testing three additional batches of LPS. Two samples of *E. coli* O127:B8 (one from Difco and the other from Sigma) and one sample of *S. marcescens* LPS (Difco) were

TABLE 2. *Amplification of PWM stimulation of human lymphocyte blastogenesis after LPS pretreament*[a]

Subject	LPS pretreatment	[^{3}H]thymidine incorporation (cpm)[b]			
		Without mitomycin C		With mitomycin C[c]	
		cpm ± SEM[d]	E/C[e]	cpm ± SEM	E/C
1	−	20,673 ± 878		11,299 ± 749	
	+	37,696 ± 2,720	1.82	10,325 ± 391	0.91
2	−	50,684 ± 1,235		15,401 ± 90	
	+	162,288 ± 1,872	3.20	10,807 ± 1,892	0.70
3	−	17,783 ± 803		9,022 ± 379	
	+	27,222 ± 180	1.53	8,287 ± 80	0.92
4	−	10,792 ± 568		6,622 ± 451	
	+	24,570 ± 742	2.28	7,048 ± 334	1.06
5	−	19,601 ± 947		11,847 ± 994	
	+	40,001 ± 529	2.04	9,821 ± 467	0.83
6	−	13,330 ± 705		8,808 ± 131	
	+	21,080 ± 1,798	1.58	9,080 ± 533	1.03
7	−	12,188 ± 464		6,158 ± 194	
	+	116,379 ± 338	9.55	6,244 ± 268	1.01
8	−	2,206 ± 105		827 ± 151	
	+	5,016 ± 227	2.27	1,097 ± 87	1.32

[a-e] See Table 1.

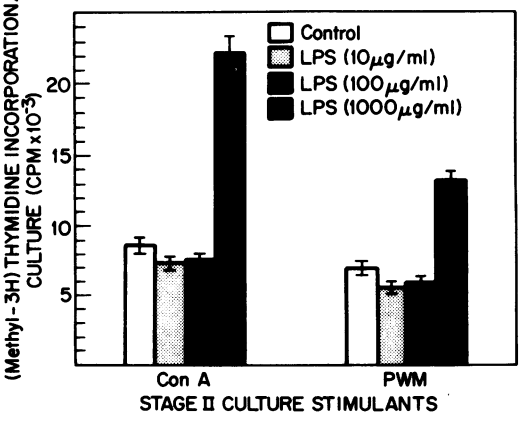

FIG. 1. *Dose dependency of B. melaninogenicus LPS in stage I (48-h preculture) lymphocyte cultures. Stage II cultures were cocultured with fresh lymphocytes, stimulated with constant amounts of ConA (5 μg/ml) or PWM (1:100), and harvested after 72 h. (Δcpm is depicted: cpm per 10^5 lymphocytes in mitogen-stimulated cultures minus cpm per 10^5 lymphocytes in unstimulated cultures.)*

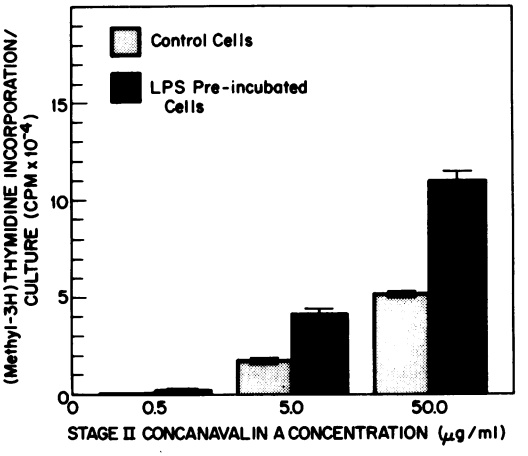

FIG. 2. *Dose-response effect of ConA in stage II coculture of preincubated and fresh lymphocytes. Stage I cells treated with B. melaninogenicus LPS (1,000 μg/ml) or media (control) for 48 h. (Data depicted as in Fig. 1.)*

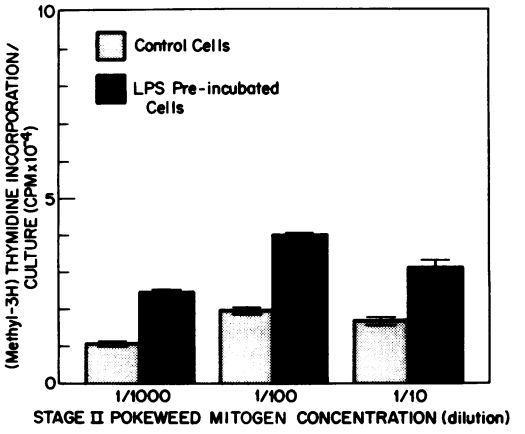

FIG. 3. *Dose-response effect of PWM in stage II coculture of preincubated and fresh lymphocytes. Stage I cells treated with B. melaninogenicus LPS (1,000 μg/ml) or media (control) for 48 h. (Data depicted as in Fig. 1.)*

compared with LPS of *B. melaninogenicus* subsp. *intermedius* prepared in our laboratory (Table 3). Although use of *B. melaninogenicus* LPS resulted in the highest amplification of blastogenesis, all four preparations of LPS were capable of mediating the effect. There were no significant variations between three independently extracted batches of LPS purified in our laboratory from *B. melaninogenicus* (not shown).

Evaluation of cells responsible for LPS enhancement. Preliminary findings (Tables 1 and 2) indicated that mitomycin C treatment of LPS-precultured lymphocytes abrogated the amplification phenomenon. Such experiments did not, however, indicate the role of the mitomycin C-sensitive cells. It was not clear whether LPS pretreatment activated a helper function which facilitated the blastogenic response of the fresh (stage II) lymphocytes to mitogen, or whether the increased DNA synthetic activity was due to a mitogen-activated helper function which facilitated a response to LPS by the stage I cells.

(i) Preliminary characterization of the amplification phenomenon. In an attempt to characterize the various cell populations participating in the amplification phenomenon, the effects of mitomycin C treatment on each cell category subsequently added to stage II were evaluated (Table 4).

In the absence of additional fresh lymphocytes, LPS alone induced a marginal blastogenic response (category 5) compared with the control precultured cells (category 3). PWM stimulation of these cells (category 5) produced a significant increase in DNA synthesis over its mitogen-stimulated control (category 3) (17,600 cpm versus 2,860 cpm).

Addition of fresh autologous cells to the LPS-precultured cells (category 8) amplified the blastogenic response when compared with the calculated values, which were based on the sum of the blastogenic responses of fresh cells (category 1) and LPS-precultured cells (category 5). Mitogenic (PWM) stimulation of these categories resulted in an additional amplification compared with the controls.

Mitomycin C treatment of the LPS-precultured cells, as previously shown (Tables 1 and 2), abrogated the amplification phenomenon almost completely in both the mitogen-stimulated and unstimulated cultures (category 10, Table 4). Mitomycin C treatment of the fresh autologous cells (categories 11 and 12) did not affect their ability to contribute to the amplification phenomenon in either the PWM-stimulated or unstimulated cultures (compare categories 8 and 12).

(ii) Identification of the role of LPS and mitogen stimulation. Although it was clear from the above experiments that an amplifying function was contributed by a cell present in the fresh cell population which influenced a cell in the LPS-precultured population, the role of each stimulant (LPS and ConA or PWM) was not clear. To identify the role of each stimulant, the following experiment was performed. Monocyte-

TABLE 3. *Comparison of the ability of different sources of LPS to amplify mitogen-induced lymphocyte blastogenesis*[a]

Cells[b]	Stimulation by[c]: ConA		PWM	
	cpm ± SD	E/C[d]	cpm ± SD	E/C
Control	3,821 ± 299		623 ± 274	
LPS (BM) precultured	7,593 ± 182	1.99	2,693 ± 848	4.32
LPS (SM) precultured	1,510 ± 392	0.39	392 ± 233	0.63
LPS (EC_1) precultured	2,790 ± 352	0.73	413 ± 52	0.67
LPS (EC_2) precultured	822 ± 195	0.23	167 ± 110	0.27
Control cells + fresh cells	6,593 ± 182		939 ± 151	
LPS (BM) precultured + fresh cells	28,422 ± 2,579	4.31	10,002 ± 537	10.65
LPS (SM) precultured + fresh cells	10,044 ± 545	1.52	1,999 ± 314	2.13
LPS (EC_1) precultured + fresh cells	16,008 ± 427	2.43	3,824 ± 442	4.07
LPS (EC_2) precultured + fresh cells	9,925 ± 764	1.51	2,793 ± 514	2.97

[a] Stage I control and LPS-treated (1,000 μg/ml) cells precultured for 48 h; stage II cultures incubated for 3 days. Each test run in triplicate.

[b] BM, *B. melaninogenicus*; SM, *S. marcescens* (Difco); EC_1, *E. coli* O127:B8 (Difco); EC_2, *E. coli* O127:B8 (Sigma).

[c] Expressed as Δcpm (cpm per 10^5 cells in mitogen-stimulated cultures minus cpm per 10^5 cells in unstimulated cultures) ± standard deviation (SD). Stimulants: ConA, 5 μg/ml; PWM, 1:100.

[d] Experimental/control.

TABLE 4. *Effect of mitomycin C treatment on the blastogenic response of stage II cocultured LPS-pretreated cells and fresh cells*[a]

Category	Response (cpm)[b]			
	Unstimulated		PWM stimulated	
	Calculated	Observed	Calculated	Observed
1. Fresh cells[c]		720		9,930
2. Fresh $cells_{mito\ C}$[d]		40		440
3. Control cells		160		2,860
4. Control $cells_{mito\ C}$		230		380
5. LPS-precultured cells		3,240		17,600
6. LPS-precultured $cells_{mito\ C}$		460		180
7. Control cells + fresh cells	880	2,440	12,790	12,950
8. LPS precultured cells + fresh cells	3,960	11,780	27,530	61,940
9. Control $cells_{mito\ C}$ + fresh cells	950	1,130	10,310	9,850
10. LPS-precultured $cells_{mito\ C}$ + fresh cells	1,180	2,035	10,110	13,030
11. Control cells + fresh $cells_{mito\ C}$	200	1,840	3,300	3,530
12. LPS-precultured cells + fresh $cells_{mito\ C}$	3,280	14,100	18,490	35,680

[a] Stage I (precultured) lymphocytes incubated with *B. melaninogenicus* LPS (1,000 μg/ml) or media (control cells) for 48 h. Stage II cultures incubated for 3 days. Individually cultured cell categories cultured at 10^5 cells/well to simulate density effects. The observed cpm is reported as one-half of the actual value in order to represent the actual contribution of each cell category to the coculture categories (50,000 cells of each category, 100,000 cells total).

[b] Expressed as [^{3}H]thymidine Δcpm (cpm per 10^5 lymphocytes in PWM [1:100]-stimulated cultures minus cpm in unstimulated cultures).

[c] Not precultured.

[d] Mitomycin C treated (50 μg/ml) for 30 min at 37°C.

depleted lymphocyte suspensions were cultured for 16 h with PWM, LPS, or culture medium alone. After washing, the various cell categories were split and half of each was exposed to 1,500 R, using a cobalt-51 source at a dose rate of 10,500 R/h to inhibit their ability to undergo a blastogenic response. Various combinations of irradiated and nonirradiated categories were then cocultured for an additional 72 h. Coculture of PWM pretreated cells with LPS pretreated cells resulted in a significantly enhanced blastogenic response (19,678 cpm versus 7,738 cpm; Table 5). Irradiation of the LPS pretreated cells abrogated the enhancement (9,644 cpm versus 7,638 cpm), whereas irradiation of the PWM pretreated cells had no effect on the enhancement. Controls for PWM ($P + P_{PWMi}$) and LPS carryover ($P + P_{LPSi}$ and $P_{PWM} + P_{LPSi}$) were negative. In addition, the optimal dose of LPS in these independently cultured experiments shifted to 10 to 100 μg/ml (not shown).

(iii) Cell type responding to LPS. From previous reports, in both murine and human studies (17), LPS has been shown to activate the B-lymphocyte subpopulation. To determine whether the cells responding to LPS in this system were in fact B cells, an experiment similar to that described above was performed. Monocyte-depleted, T- and B-enriched lymphocyte fractions were cultured with LPS (100 μg/ml)

TABLE 5. *Characterization of the role of LPS and mitogen in the amplification phenomenon*

Category[a]	[*methyl*-^{3}H]thymidine incorporation (cpm)	
	Calculated	Observed
P		295
P_i		66
P_{PWM}		9,866
P_{PWM_i}		758
P_{LPS}		363
P_{LPS_i}		146
$P + P_i$	361	587
$P + P_{LPS}$	658	734
$P + P_{LPS_i}$	441	179
$P_i + P_{PWM}$	9,932	7,738
$P_{LPS_i} + P_{PWM}$	10,012	9,644
$P + P_{PWM_i}$	1,052	2,004
$P_{LPS} + P_{PWM_i}$	1,121	7,025
$P + P_{PWM}$	10,161	7,738
$P_{LPS} + P_{PWM}$	10,229	19,678

[a] Lymphocytes (2×10^6/ml) were cultured in the presence of PWM (1:100 stock) or LPS (100 μg/ml) for 16 h. After washing, the cells were irradiated (1,500 R) as described. The indicated cell categories were cocultured for an additional 72 h. Six hours before termination, 2 μCi of [*methyl*-^{3}H]thymidine was added to each culture to assess DNA synthetic activity. Precultured (P) cell categories are indicated by subscript: LPS or PWM. No subscript indicates culture medium preculture alone. ("*i*" indicates irradiated-cell category).

for 16 h. A monocyte-depleted lymphocyte fraction was simultaneously cultured with PWM (1:100). After washing, this fraction was irradiated (1,500 R) and added to an equal number (100,000) of LPS-precultured cells, which were then cultured for an additional 72 h. Only the LPS-pretreated B lymphocytes demonstrated the amplification phenomenon (Table 6).

(iv) Cell type providing helper activity. To identify the source of the helper activity, lymphocytes were separated in B and T subpopulations as previously described, cultured with PWM (1:1,000) for 16 h, and irradiated. These cells were added to LPS-precultured, monocyte-depleted lymphocytes and cultured for an additional 72 h. The source of helper activity was localized in the T-cell preparations (Table 7).

DISCUSSION

The presence of regulatory lymphocytes in the blood of normal human subjects has been extensively documented (10, 11, 22, 24, 25). A suppressive subpopulation of these cells can be activated by preculture with the mitogens ConA or phytohemagglutinin (PHA) (4, 6, 11, 25). These cells have a maximal suppressive effect on the blastogenic response of lymphocytes stimulated with such T-cell activators as ConA, PHA, and the allogeneic mixed lymphocyte reaction.

TABLE 6. *Identification of the lymphocyte subpopulations responding to LPS pretreatment*[a]

Category	[*methyl*-^{3}H]thymidine incorporation (cpm ± SD)[b]	
	+P_i[c]	+P_{PWM_i}[d]
Monocyte depleted	247 ± 29	1,037 ± 137
Monocyte depleted precultured LPS	1,041 ± 385	5,043 ± 243
T cells	274 ± 274	1,374 ± 526
T cells, LPS precultured	319 ± 57	2,898 ± 442
B cells	285 ± 89	1,297 ± 341
B cells, LPS precultured	297 ± 76	19,753 ± 729

[a] After monocyte depletion, lymphocytes were resolved into B- and T-cell subfractions. Monocyte-depleted B and T cells were cultured with LPS (100 μg/ml) for 16 h. Concurrently, monocyte-depleted cells were cultured with PWM (1:100 stock). After the preculture, the cells were washed and the PWM-stimulated cells were irradiated (1,500 R). The appropriate cell categories were then cocultured as shown for an additional 72 h. Six hours before termination, 2 μCi of [*methyl*-^{3}H]thymidine was added to each culture to assess DNA synthetic activity.

[b] SD, standard deviation.

[c] Irradiated, medium-precultured, monocyte-depleted lymphocytes.

[d] Irradiated, PWM-precultured, monocyte-depleted lymphocytes.

TABLE 7. *Identification of lymphocyte subpopulations providing helper activity to LPS-pretreated cells*[a]

Helper cell category[b]	[*methyl*-^{3}H]thymidine incorporation (cpm)	
	+P[c]	+P_{LPS}
Monocyte depleted$_i$	73 ± 03	243 ± 633
Monocyte depleted-PWM$_i$	8,302 ± 971	36,173 ± 2,633
T cell$_i$	43 ± 41	363 ± 84
T cell$_{PWM_i}$	789 ± 494	11,192 ± 2,201
B cell enriched$_i$	366 ± 79	1,478 ± 86
B cell enriched$_{PWM_i}$	1,478 ± 327	1,853 ± 104

[a] After monocyte depletion, lymphocytes were separated into B- and T-subpopulations. Monocyte-depleted B and T cells were cultured with PWM (1:1,000) for 16 h, washed, and irradiated (1,500 R). Simultaneously, monocyte-depleted lymphocytes were cultured with LPS (100 μg/ml) and washed. The above cell categories were cocultured for an additional 72 h. Six hours before termination, 2 μCi of [*methyl*-^{3}H]thymidine was added to each culture to assess DNA synthetic activity.

[b] *i*, Mock preculture with medium followed by washing and irradiation; PWM$_i$, preculture with PWM (1:1,000) followed by washing and irradiation.

[c] P and P_{LPS}, precultured with medium control and with LPS (100 μg/ml), respectively.

LPS has been described as being incapable of inducing blastogenesis in the peripheral blood lymphocytes of normal human subjects (21). However, it has been shown to mediate a synergistic response when added to lymphocytes simultaneously with ConA or PHA (23). In those studies, the blastogenic response to the cocultured compounds was greater than the sum of the responses of the individually cultured stimulants. By using an LPS preculture technique to separate the LPS and mitogen activation steps, we have attempted to construct a model with which further evaluations of LPS-associated amplification or synergy could be studied.

Our studies indicated that such amplification associated with LPS is due to the B-lymphocyte response to that substance which is facilitated by a helper effect induced by a second stimulant. The possibility that the augmented response was merely due to carryover of LPS from stage I cultures is minimal since irradiated LPS-pretreated cells had no influence when cocultured with PWM pretreated cells, and other carryover controls described in Results were negative.

Amplification of lymphocyte blastogenesis can be mediated through a variety of cellular mechanisms (7). Enhancement of DNA synthetic activity may represent a functional change in immunoregulation, which can be manifested either as expression of a helper activity or as an

inactivation of suppressor cell function. In the present experiments, mitomycin C treatment or gamma irradiation of mitogen-precultured cells was shown to have no abrogating effect on the helper activity.

Mitogen-associated amplification of the response was demonstrated in the absence of additional fresh mononuclear cells; however, the absolute magnitude of the response by no means approached that elicited when cocultured with fresh cells. This finding supports the presence of active helping lymphocytes, rather than a loss of suppressive cells. Based on these findings, we propose that during LPS preculture a B-lymphocyte subpopulation is triggered. However, a response to LPS does not occur unless a helping activity is provided by the presence of a costimulant which activates an accessory cell.

Reports by MacDermott et al. (16) have demonstrated the requirement for a radioresistant T-cell population important in the human B-lymphocyte response to mitogens. These T cells may represent the amplifying cells in our model. In studies by DuBois et al. (5), irradiated autologous or allogenic cells were shown to be capable of reconstituting in vitro proliferative responses to tetanus toxoid even if the reconstituting cells were themselves unresponsive to the stimulant. Such findings indicate the existence of a population of regulatory cells capable of providing complementary signals to cells that are unable to respond to substances such as LPS. Inability to activate such helper cells would explain the difficulty in demonstrating a human lymphocyte response to such classic murine B-cell polyclonal cell activators as LPS (21). Miller et al. (17) have recently reported the stimulation of human peripheral blood lymphocytes with purified LPS. Their studies suggested that a T-helper cell may be required to obtain stimulation. Our investigations appear to confirm the requirement of a helper activity with LPS. Current studies in progress in our laboratory are attempting to further elucidate the nature of the helper phenomenon.

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