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J Immunol (1980) 124 (2): 635–640.
https://doi.org/10.4049/jimmunol.124.2.635

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0022-1767/80/1242-0635$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 124, No. 2, February 1980
Printed in U.S.A.

LIPOPOLYSACCHARIDE-INDUCED IMMUNOMODULATION OF THE GENERATION OF CELL-MEDIATED CYTOTOXICITY

I. Suppression of the Development of Cytotoxic Lymphocytes[1]

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Bacterial lipopolysaccharide from a variety of Gram-negative organisms suppresses the development of cytotoxic killer cells in the murine MLC. Cytotoxic T lymphocytes were generated *in vitro* by incubating BALB/c responder spleen cells with irradiated C57BL/6 stimulator cells for 5 days in mixed lymphocyte culture (MLC). The addition of LPS at the initiation of MLC suppressed killing of ^{51}Cr-labeled target cells in a dose-dependent manner. LPS was active only during the afferent phase of CMC, since it did not interfere with the efferent phase of the assay. Furthermore, timed addition and timed removal studies suggested that the presence of LPS during the first 48 hr of MLC was critical for maximal suppression of CMC. Lipid A extracted from LPS, which had been shown to be highly suppressive when added to the sensitization phase of the CMC assay, was also inhibitory. Moreover, when LPS was added to MLC in the presence of tritiated thymidine, the proliferative activity of the responder cells increased markedly after 72 hr of culture. These data suggest that LPS, a known B cell mitogen, can modulate the complex sequence of cellular interactions that leads to the generation of cell-mediated cytotoxicity.

Many immunoregulatory effects of lipopolysaccharide (LPS) from Gram-negative bacteria have been described. LPS has been shown to be selectively mitogenic for murine bone marrow-derived (B) lymphocytes (1). As a polyclonal activator, LPS augments immunoglobulin synthesis to a variety of seemingly unrelated antigens (2). Furthermore, LPS acts as an adjuvant for antibody synthesis when given with antigen (3). The administration of LPS shortly after antigen will abrogate a state of specific antibody unresponsiveness to that antigen (4). Antibody responses to haptens on isologous erythrocyte carriers can also be induced in mice with the concomitant administration of LPS (5).

Some immunosuppressive effects have also been ascribed to LPS. Spleen cells taken from mice that received an i.p. injection of LPS and mixed with normal spleen cells and sheep red blood cells (SRBC) had a decreased primary antibody response *in vitro* to the SRBC compared to responses of spleen cells from mice that received no LPS (6). The simultaneous addition of LPS and antigen to spleen cells can inhibit *in vitro* (7) as well as *in vivo* (8) primary antibody production. Studies have shown that LPS also suppresses *in vitro* secondary responses in murine spleen cells when added to cultures 12 and 48 hr after antigen (9).

LPS can also modulate the functions of thymus-derived lymphocytes (T cells). Anti-tumor immunity (10), graft *vs* host reaction (11), alloantigen-stimulated cell-mediated cytotoxicity (CMC)[4] (12), and allograft rejection (13) are T cell-associated activities modulated by LPS. The simultaneous addition of LPS and concanavalin A (Con A) to murine thymocytes increases synergistically their mitogenic responses (14). Similar observations have been made when human peripheral blood SRBC rosette-forming T cells were activated by Con A or PHA and LPS (15). Furthermore, Norcross and Smith (16) have shown that a subpopulation of mouse thymus cells act in synergy with B cells to modulate their proliferative responses to LPS. We have chosen to examine the immunomodulatory effects of LPS on the generation of murine T CMC *in vitro*. The purpose of this report is to show that LPS, in a dose-dependent manner, inhibits the generation of CMC in the murine mixed lymphocyte culture (MLC).

MATERIALS AND METHODS

Mice. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice of both sexes were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used at 2 to 5 months of age.

Lipopolysaccharide (LPS). LPS extracted from a variety of Gram-negative bacteria was purchased from Difco Laboratories, Detroit, Mich. LPS was dissolved in water, and solubility was enhanced by incubation in an ultrasonic water bath for 2 min. The solution was then boiled in water for 2 min before Millipore filter sterilization.

Extraction of lipid A from bacterial LPS. Lipid A was extracted from LPS by mild acid hydrolysis with minor modification of the technique Galanos *et al.* (17). Briefly, 100 mg of LPS were suspended in 6 ml of water; 6 ml of 0.2 N acetic acid were added. The suspension was boiled for 4 hr and then centrifuged (13,000 × G, 25 min). The pellet was washed three

Received for publication July 19, 1979.
Accepted for publication October 30, 1979.
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[1] This work was funded in part by American Cancer Society Grant IN-13-R-25 and by Minnesota Medical Foundation Grant CRF-36-79. Dr. Vallera was supported by Training Grant 1-T32-CA-09138 from the National Cancer Institute, National Institutes of Health.
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[4] Abbreviations used in this paper: CMC, cell-mediated cytotoxicity; CTL, cell-mediated lympholysis.

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times with water, once with acetone, and then dried in a desiccator above $CaCl_2$. Since lipid A is relatively insoluble, it was linked to bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, Mo.). Therefore, 20 mg of the dried lipid A pellet were suspended in 10 ml of water and heated to 100°C for 2 min. Also, 20 mg BSA were dissolved in distilled water in a separate tube. The solutions were mixed and heated 100°C for 2 min. The 1:1 BSA:lipid A mixture was then lyophilized and stored at 4°C.

Cytotoxicity. The murine MLC generation of CMC has been previously reported (18). Briefly, 4×10^6 responder spleen lymphocytes (BALB/c) were incubated with 1×10^6 irradiated (2000 R) stimulator splenocytes (C57BL/6) in 16-mm well tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.) in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS) (Reheis Chemical Co., Phoenix, Ariz.), penicillin-streptomycin, 5×10^{-5} M 2-mercaptoethanol, nonessential amino acids, sodium pyruvate, and L-glutamine.

After 5 days culture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, cells from six replicate wells were pooled, washed, and incubated in triplicate at 37°C with ^{51}Cr-labeled EL4 tumor target cells of C57BL/6 origin. A ^{51}Cr-labeled BALB/c tumor (LSTRA) was used as a specificity control for all experiments. Both lymphoma tumors, maintained by ascites passage, were labeled with 0.2 mCi ^{51}Cr (New England Nuclear, Boston, Mass.) for 60 min at 37°C. 5×10^4 ^{51}Cr-labeled target cells and various numbers of effector cells in triplicate were incubated in a volume totaling 200 μl in V-bottomed microtiter plates (Linbro Chemical Co.) for 4 hr at 37°C. Target cells alone were incubated to determine the spontaneous release of ^{51}Cr isotope. The plates were centrifuged for 10 min at 700 × G, and 100 μl supernatant were removed from each well and assayed for radioactivity in a Beckman Biogamma II counter. Percent cytotoxicity was then calculated:

$$\% \text{ cytotoxicity} = \frac{(\text{Experimental cpm}) - (\text{spontaneous cpm})}{(\text{Maximum cpm}) - (\text{spontaneous cpm})} \times 100$$

The data in each figure are representative of at least three replicate experiments; p values were calculated by the Student *t*-test.

Assay for proliferation. Lymphocytes were cultured in Eagle's MEM in 16 mm wells, and 2 μCi tritiated thymidine (specific activity 15 Ci/mmole) in 50 μl MEM were added to each well 24 hr before harvest. MEM was supplemented with 10% pooled human serum for these studies. Cultures were collected by precipitation onto glass fiber filters with a 12-place automated harvester. The filters were dried in scintillation vials, and 3.7 ml of scintillation fluid were added. The vials were sealed and the samples were assayed for ^{3}H activity in a Beckman LS 300 scintillation counter.

RESULTS

Effect of LPS or lipid A on the generation of CMC in murine MLC. Data in Table I show that when added at the initiation of MLC, 75 and 100 μg LPS extracted from a variety of Gram negative bacteria by either the Westphal or Boivin procedures (19), markedly inhibit the generation of CMC. Moreover, lipid A extracted from *Shigella flexneri* LPS suppressed the generation of cytotoxicity to a greater degree than the LPS itself since lipid A (37 μg/ml) reduced cytotoxic levels to the same degree as twice the amount of LPS (75 μg/ml). Significant inhibition of CMC was evident when target cells were incubated with multiple ratios of effector cells. No cytotoxicity was detected in either control or treated groups when cells syngeneic to the responding BALB/c spleen cells, LSTRA tumor cells, were used as targets.

Dose-dependent effect of LPS on the generation of CMC in murine MLC. Significant inhibition ($p < 0.001$) of CMC was noted when 25 μg/ml LPS from *Escherichia coli* 0111:B4 (Fig. 1*A*) or *S. flexneri* (Fig. 1*B*) were added to the MLC, with increasing inhibitory effects noted at 50, 75, and 100 μg/ml. No significant effect on the generation of CMC was noted when 1 and 5 μg/ml LPS were added to MLC. In all instances, sup-

TABLE I

The suppressive effect of LPS and lipid A on the generation of CMC[a]

Bacteria	LPS[b]	Extraction Method	% Cytotoxicity at Different Effector: Target Ratios[c]			
			EL4			LSTRA
			8:1	4:1	2:1	8:1
	μg					
	0		74 ± 2	73 ± 3	60 ± 4	3 ± 0.2
Salmonella abortus equi	100	Westphal	43 ± 2[d]	25 ± 1[e]	14 ± 1[e]	1 ± 0.1
Serratia marcescens	100	Westphal	15 ± 1[e]	8 ± 0.5[e]	3 ± 0.2[e]	1 ± 0.1
Escherichia coli	100	Westphal	1 ± 0.1[e]	1 ± 0.2[e]	0 ± 0.1[e]	0 ± 0.1
	0		67 ± 4	64 ± 5	53 ± 2	4 ± 0.2
Salmonella typhosa	75	Boivin	21 ± 2[e]	11 ± 0.5[e]	6 ± 0.3[e]	−6 ± 0.1
Salmonella typhimurium	75	Boivin	17 ± 2[e]	9 ± 1[e]	5 ± 0.5[e]	−6 ± 0.1
Serratia marcescens	75	Boivin	10 ± 1[e]	5 ± 0.3[e]	2 ± 0.1[e]	−6 ± 0.2
	0		63 ± 5	58 ± 4	45 ± 3	2 ± 0.2
Shigella flexneri	75	Westphal	16 ± 2[e]	10 ± 1[e]	5 ± 0.2[e]	1 ± 0.1
Shigella flexneri lipid A[f]	37	Mild acid hydrolysis	20 ± 2[e]	7 ± 1[e]	3 ± 0.2[e]	2 ± 0.1

[a] BALB/c responders were cultured with irradiated (2000 R) C57BL/6 splenocytes for 5 days. LPS or lipid A were added at the initiation of MLC. Effector cells were harvested and incubated with ^{51}Cr-labeled EL4 and LSTRA cells in a 4-hr assay.

[b] LPS was obtained from Difco Laboratories and added to cultures at 75 or 100 μg LPS or lipid A/ml medium. Lipid A was extracted from *Shigella flexneri* LPS by mild acid hydrolysis.

[c] Values are mean ± S.D. of triplicate samples.

[d] $p < 0.01$ as calculated by the Student *t*-test.

[e] $p < 0.001$ as calculated by the Student *t*-test.

[f] Lipid A was conjugated to BSA (1:1) to facilitate its solubility. Addition of BSA alone to MLC had no effect on cytotoxicity (data not shown).

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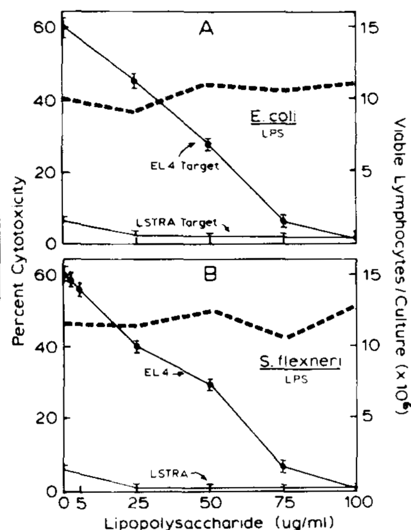

Figure 1. BALB/c responder splenocytes, (4×10^6), were cultured with irradiated (2000 R) C57BL/6 splenocytes, (1×10^6), for 5 days in MLC. Varying amounts of LPS from *E. coli* (*A*) and *Shigella flexneri* (*B*) were added at the initiation of culture. Cytotoxicity was measured with ^{51}Cr-labeled EL4 and LSTRA target cells in a 4-hr assay. Viability of the effector cells following MLC was determined by trypan blue dye exclusion. Each test value represents mean percent cytotoxicity ± S.D. of triplicate samples at an effector to target ratio of 4:1 ($p < 0.001$ in the presence of 25, 50, and 75 μg LPS/ml).

TABLE II

Effect of LPS on the efferent phase of the CMC assay[a]

Target	LPS Present[b]	% Cytotoxicity		
		8:1	4:1	2:1
EL4		76 ± 9	71 ± 1	55 ± 1
LSTRA		0 ± 0.4		
EL4	Efferent phase[c]	76[d] ± 4	70[d] ± 3	54[d] ± 3
EL4	Afferent phase[e]	4[f] ± 0.6	1[f] ± 0.2	0.1[f] ± 0.3

[a] BALB/c responders, 4×10^6, were incubated with irradiated (2000 R) C57BL/6 stimulators, 1×10^6, for 5 days. LPS was added either at the initiation or after MLC. The effector cells were harvested and cultured with ^{51}Cr-labeled EL4 and LSTRA target cells in a 4-hr release assay. Values are mean percent cytotoxicity ±S.D. of triplicate samples.

[b] 100 μg *E. coli* LPS/ml media.

[c] LPS is added to the alloantigen-sensitized BALB/c effector cells after MLC.

[d] $p < 0.988$ compared to untreated controls (calculated by the Student *t*-test).

[e] LPS is added at the initiation of the MLC.

[f] $p < 0.001$ compared to untreated controls.

pression of CMC by LPS was not associated with a decrease in viability (as determined by exclusion of trypan blue from the viable effector cells harvested from the MLC).

Comparative effects of LPS on the afferent and efferent phase of the murine MLC generation of CMC. The possibility existed that LPS was being carried over from the MLC and was acting during the efferent phase of the CMC assay (i.e., during the 4-hr exposure of responder cells to ^{51}Cr-labeled target cells.) Data in Table II, however, show that the addition of a suppressive dose of LPS (100 μg/ml) to the efferent phase of the assay did not affect CMC. Therefore, the inhibitory action on CMC by LPS occurred during the afferent phase of the generation of CMC.

Effect of LPS on the murine MLC when added or removed at different intervals. To determine the time at which LPS exerted its inhibitory effect on the generation of CMC, LPS was added to each culture at 24-hr intervals after the initiation of the MLC. The data in Figure 2 show that a suppressive dose of LPS (100 μg/ml) significantly ($p < 0.001$) inhibited CMC when added 24 and 48 hr after the initiation of MLC. However, if LPS was added later, the reduction of cytotoxicity was not as dramatic.

In timed removal studies (Fig. 3), LPS was added to all cultures at the start of MLC. Cells were washed at daily intervals and then returned to culture. If LPS was washed from the cultured cells at 24 or 48 hr, suppression of CMC was not as

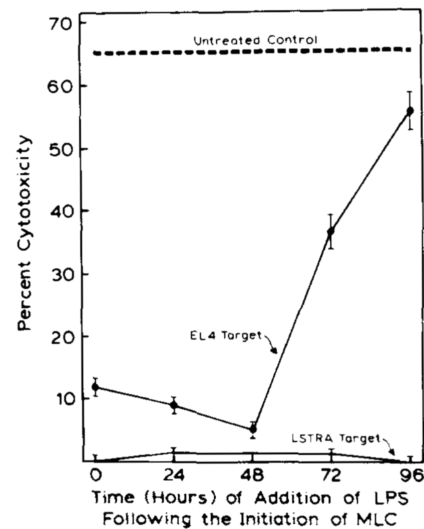

Figure 2. BALB/c splenocytes (4×10^6) were cultured with irradiated (2000 R) C57BL/6 splenocytes, (1×10^6), in MLC for 5 days. *E. coli* LPS (100 μg/ml media) was added to groups of lymphocytes in MLC at 24-hr intervals. Effector cells were then harvested and incubated with ^{51}Cr-labeled EL4 and LSTRA target cells at an effector to target ratio of 4:1. Values represent mean percent cytotoxicity ± S.D. of triplicate samples. $p < 0.001$ when LPS was added at 0, 24, or 48 hr. $p < 0.02$ when LPS was added at 72 or 96 hr.

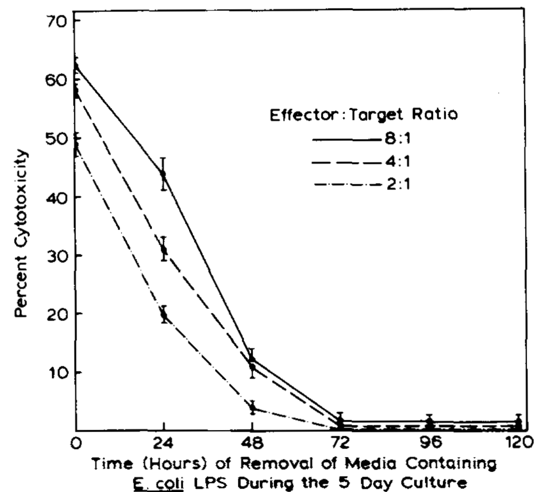

Figure 3. BALB/c responder splenocytes (4×10^6) were cultured with irradiated (2000 R) C57BL/6 splenocytes (1×10^6) for 5 days in MLC. *E. coli* LPS (100 μg/ml media) was added to all test samples at the initiation of culture. Samples were removed at 24-hr intervals, washed, and returned to culture. After MLC, effector cells were incubated with ^{51}Cr-labeled EL4 target cells at effector to target ratios 8:1, 4:1, and 2:1. Test values represent mean percent cytotoxicity ± S.D. of triplicate samples.

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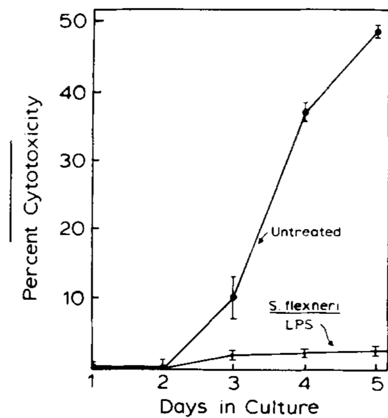

Figure 4. BALB/c responder splenocytes, (4×10^6), were cultured with irradiated (2000 R) C57BL/6 (1×10^6) splenocytes for 5 days in MLC. *Shigella flexneri* LPS (75 μg/ml media) was added at the initiation of culture. Samples were harvested at 24-hr intervals and incubated with ^{51}Cr-labeled EL4 target cells. Each test value represents the mean percent cytotoxicity ± S.D. of triplicate samples at an effector to target ratio of 4:1.

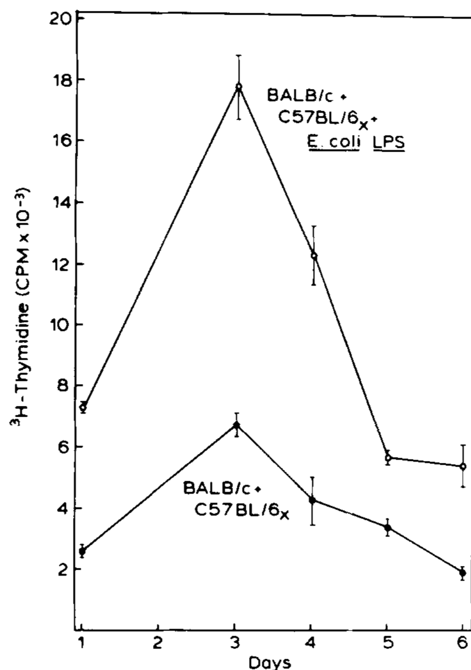

Figure 5. BALB/c responder cells (4×10^6) were incubated with C57BL/6 stimulator cells (1×10^6) in MLC. *E. coli* LPS (75 μg/ml media) was added at the initiation of culture. The cells were harvested at 24-hr intervals. Label (2 μCi tritiated thymidine) was added 18 hr before harvest. Cultures were harvested by precipitation onto glass fiber filters and samples assayed for ^{3}H activity. Each point represents the mean cpm ± S.D. of triplicate samples. The increase of ^{3}H uptake is significantly greater ($p < 0.001$) when LPS was added to the MLC.

great as if LPS was removed later. These experiments suggested again, that LPS exerted its maximal effect within the first 48 hr of MLC.

Effect of LPS on the kinetics of the generation of CMC. To determine if the LPS-induced inhibition of the generation of CMC was simply due to a change in the kinetic pattern of the response, CMC was measured at 24-hr intervals over the 5-day culture period. Data in Figure 4 show that the generation of CMC was completely inhibited by LPS over the entire 5-day culture period compared to cultures in which no LPS was added. Thus, the observed suppression of CMC by LPS was not due to a shift in time of peak cytotoxicity over the 5-day culture time.

The proliferative activity of cells in MLC in the presence of LPS. BALB/c responder cells were cultured with irradiated C57BL/6 stimulators and pulsed at daily intervals with ^{3}H-thymidine (^{3}H-TdR) (Fig. 5). The uptake of labeled thymidine occurred earlier and was significantly greater ($p < 0.001$) when 75 μg *E. coli* LPS/ml were added to MLC compared to untreated controls. ^{3}H-TdR uptake increased over the first 3 days, reaching a maximum on day 3. These data suggest that LPS enhances MLR activity and is stimulatory toward a subpopulation of lymphocytes present in the responder fraction. Therefore, LPS could be suppressing the generation of CMC through a population of activated B lymphocytes.

DISCUSSION

Because of its well-known stimulatory affects on B cells, LPS has been used as an effective tool in studying the immunomodulation of the humoral response. Persson (6) showed that addition of LPS to lymphocytes cultured with a thymic dependent antigen (sheep red blood cells) markedly suppressed primary antibody responses compared to untreated control cultures. This affect was due to the presence of suppressor cells. The suppressor activity was not removed by treatment of the cells with anti-Θ plus complement (C) nor by removal of macrophages by treatment with carbonyl iron powder suggesting that the suppressors were not of T cell or macrophage origin. Interestingly, Persson (6) also showed that although LPS-activated cells responded mitogenically to purified protein derivative (PPD) and LPS (both polyclonal B cell activators), the responses generated to Con A, a T cell mitogen, were much different. This suggested that LPS-induced suppressor cells acted on T lymphocytes.

Walker and Weigle (9) reported that LPS also suppressed *in vitro* secondary antibody responses in mice to certain protein antigens. Consistent suppression was dependent on the time of LPS addition. Peak suppression occurred when LPS was present in the cultures within the first 48 hr. Later addition of LPS was less effective. These findings were identical to those described by our laboratory when LPS was added to MLC of murine splenocytes (Fig. 2), and cytotoxicity was suppressed in a similar manner. This suggests that although these two systems measure different functions (humoral *vs* cellular immunity), it is quite possible that a cell type common to the generation of responsiveness in both systems is effected by the addition of LPS, namely helper T cells.

Bacterial LPS is known to elicit an effect on T lymphocytes. Armerding and Katz (20) studied the effects of LPS on *in vitro* primary and secondary antibody responses to soluble hapten-protein conjugates and to particulate and soluble sheep erythrocyte antigens. Their results showed that the action of LPS was directed at B lymphocytes or T lymphocytes depending on culture conditions. In the absence of antigen, LPS acted predominantly on B lymphocytes. When antigen was present, LPS significantly influenced specific helper T cell function. This suggests that a major biologic effect of LPS on antibody production is mediated through its influence on antigen-specific helper T cell function. Other studies (21, 22) have shown that the potent *in vivo* adjuvant effect of LPS also involves a stimulated helper function.

The generation of cytotoxic T lymphocytes in the murine CMC assay that we employ for these studies involves the

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interaction of certain T cell subpopulations. Cantor and Boyse (23) have shown that the maturation of Lyt-2,3 T killer cells is regulated by helper T cells bearing Lyt-1 surface markers. Lyt-1 cells proliferate maximally in response to antigen whereas Lyt-2,3 cells exhibit minimal proliferation (24). Bach and coworkers (25–27) have shown that cytotoxic lymphocytes (presumably Lyt-2,3 cells) recognize the H-2K and H-2D region-associated "cytotoxic determinant" (CD) antigens on target cells in the cell-mediated lympholysis (CTL) assay. Another T cell population (presumably Lyt-1 cells) respond to H-2I region determinants resulting in proliferation and a helper effect in the generation of CTL.

The exact nature in which these helper T cells assist in the generation of cytotoxic cells in unknown. However, disparity between the H-2K or H-2D regions and the H-2I region results in a strong proliferative and cytotoxic response suggesting a strong help signal. Alternatively, when stimulators differ from responders only at the H-2D or H-2K region, both proliferative and cytotoxic responses are very weak (28). In our studies, responder-stimulator combinations differed over the entire H-2 complex (BALB/c: H-2^d, C57BL/6: H-2^b). These major histocompatibility differences, in the presence of selected FCS, provided optimal conditions for the generation of help and development of CTL since a high degree of target cell killing was obtained at relatively low (4:1, 2:1) effector:target ratios.

Narayanan *et al.* (29) have shown that LPS can substitute for or provide a helper effect in a murine MLC-induced cytotoxicity assay in which the stimulatory signal is suboptimal. Relative to our system, small amounts of LPS (5 μg/ml) enhanced cytotoxic responses to minor histocompatibility antigen differences in congenic mouse strains. (This amount of LPS had no effect on the generation of CMC in our system. In both systems the commercial source of LPS was the same.) They cultured cells in 1% normal mouse serum, which is not in itself stimulatory. Cytotoxicity was assayed by using ^{51}Cr spleen cell blasts (PHA-induced) at relatively high effector:target cell ratios (50:1, 40:1). The repeated augmentation of alloantigen stimulated cytotoxicity by LPS as reported by Narayanan *et al.* (29) contrasts with our results. However, the results are not surprising when considered in light of the differences in experimental systems. It may be hypothesized that in such a suboptimal system, when compared to ours, immunomodulatory agents such as LPS and poly A:U might provide a helper signal, as it does in humoral systems (5, 20), resulting in augmented cytotoxicity compared to the amount of cytotoxicity generated in the absence of such agents. However, in our system, in the presence of selected FCS, we might conclude that there is already an optimal or maximal generation of CMC and that immunomodulatory agents such as LPS provide, by an unknown mechanism, a suppressive signal. It may be speculated that too much help in the form of the amounts of LPS used in our system might result in an overall inhibitory effect. In both these systems, as in the humoral systems that were discussed previously, it appears that LPS, depending on culture conditions, can modulate the immune response. Whether certain immunologic functions such as cytotoxicity or antibody-production are augmented or suppressed may be dependent on the effect of LPS on T helper cells. However, these studies certainly do not rule out the involvement of other cell types.

In the accompanying paper, we show that LPS does not appear to act directly on T lymphocytes. LPS-induced suppression of CMC is linked to proliferating B cells (as was suggested by the ability of Lipid A to suppress CMC in this paper). Studies are described that indicate that B lymphocytes participate in the regulation of T cell-mediated immune activities. The mechanism by which LPS-stimulated B cells modulate cytotoxicity is also examined.

Acknowledgments. The authors wish to acknowledge Drs. P. R. Narayanan and F. H. Bach for their comments on this manuscript, and Ms. Jean Ohman for her excellent secretarial contributions.

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