0019-9567/78/0019-0071$02.00/0
INFECTION AND IMMUNITY, Jan. 1978, p. 71-78
Vol. 19, No. 1

Printed in U.S.A.

Mitogenic Activity of Bacterial Lipopolysaccharides In Vivo: Morphological and Functional Characterization of Responding Cells

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Received for publication 22 July 1977

The in vivo effect of bacterial lipopolysaccharides (LPS) on mouse spleen cell subpopulations was investigated. Intravenous administration of LPS resulted in marked enlargement of the spleen, accompanied by increased cellular proliferation and enhanced nucleated cell recoveries. At least two morphologically distinct cell types appeared to be targets for LPS. Polymorphonuclear leukocytes accumulated rapidly with a relatively minor degree of cell division. In contrast, a substantial proportion of splenic lymphocytes transformed into large lymphocytes and blast cells which actively incorporated [^{3}H]thymidine. Proliferating cells were identified as bone marrow-derived (B) lymphocytes by their ability to form C3-dependent rosettes and to synthesize immunoglobulin. These cellular responses were not antigenically induced, since LPS derived from mutants lacking the polysaccharide moiety gave similar results. Thus, splenic B lymphocytes appear to interact and respond to LPS in vivo in the same manner as observed in vitro. These data suggest that the capacity of LPS to directly activate B lymphocytes, initiate cellular proliferation, and induce immunoglobulin production by bone marrow-derived cells in vivo may contribute to its adjuvant activity.

The lipopolysaccharides (LPS) or endotoxins of gram-negative bacterial species induce morphological transformation, cell division, and immunoglobulin synthesis by bone marrow-derived (B) lymphocytes in vitro (4, 5, 15, 27, 28, 35). In vivo, LPS has an adjuvant effect, enhancing B-cell responses to a variety of antigens (11, 37). In vitro studies have led to the suggestion that this adjuvant effect is due to the direct mitogenic effect of LPS on precursors of specific antibody-producing cells. The lipid A component may enable LPS to bind to B-lymphocyte membranes, thereby delivering an induction signal similar to the stimulus normally supplied by thymus-derived (T) lymphocytes (3, 16, 29, 46, 47). Results from in vivo investigations, however, suggest that the adjuvant effect of LPS is not due entirely to direct activation of B lymphocytes, but may involve macrophages and/or T lymphocytes as well. Incubation of macrophages with soluble antigen and endotoxin augments antibody responses when these cells are injected into mice (40). Furthermore, LPS fails to potentiate antibody formation in mice that have been depleted of T lymphocytes (2). Using an adoptive transfer system, Hamaoka and Katz provided evidence that LPS administration enhanced helper T-cell activity without increasing hapten-specific B-cell responses (17).

Proliferation of cells other than B lymphocytes also results from the administration of LPS in vivo. LPS enhances hemopoietic colony formation in the spleens of irradiated mice with participation by myeloid, erythroid, and megakaryocytic cell lines (38, 39). Mitosis of monocytes and macrophages has also been observed after injection of LPS (14). To date, a direct effect on proliferation of T lymphocytes has not been demonstrated conclusively, but thymic involution and the appearance of blast cells in the thoracic duct have been shown to occur within hours after LPS administration (19, 21). Because several of these cell types play a direct or indirect role in antibody synthesis, their responses might contribute to the adjuvant activity of LPS in vivo (31). It is possible that LPS acts by inducing the proliferation and/or mobilization of several cell types from primary and secondary lymphoid tissues. Since the mouse spleen contains all cells required for the production of antibody and is also a source of hemopoietic stem cells, we have chosen to employ morphological and functional techniques to identify spleen cells that respond to LPS in vivo.

MATERIALS AND METHODS

Animals. A, BALB/c, C57Bl/6, and DBA/2 mice (Jackson Laboratories, Bar Harbor, Me.), 6 to 8 weeks of age, were housed in groups of 10 and maintained on acidified chlorinated water and laboratory chow ad libitum.

Endotoxin. *Salmonella typhimurium* 7 was obtained from M. Herzberg, Department of Bacteriology, University of Hawaii, and grown in glucose salts medium for 18 h at 37°C. LPS was extracted from Formalin-fixed cells by the phenol-water procedure (48), suspended in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), and stored at 4°C until used. Additional LPS preparations were extracted from three rough strains of *S. typhimurium,* each of which possessed a specific defect in biosynthesis of LPS polysaccharides. These strains are designated TV 119 (an Ra mutant of *S. typhimurium* LT-2), SL 684 (a mutant of *S. typhimurium* LT-2, chemotype Rc, which lacks uridine 5'-diphosphate-galactose-4-epimerase), and SL 1102 (chemotype Re). The original sources and conditions for growth of these strains have been described previously (9); the chemical composition of their LPS is in Table 7.

Activation of spleen cells in vivo. Mice received 50 μg of LPS intravenously (i.v.) at the start of each experiment. At the same time or at 24-h intervals thereafter, LPS-treated or control mice were injected i.v. with 25 μCi of [^{3}H]thymidine (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). At 24 h after each pulse, spleens were harvested from three mice of each group, and single-cell suspensions were prepared (1). Portions of each spleen cell suspension were used to determine (i) total nucleated cell counts, (ii) incorporation of tritiated thymidine, and (iii) differential classification by light microscopy.

Cell quantitation and viability determination. Nucleated cells were counted with a model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.). Viability was determined by trypan blue exclusion.

Measurement of deoxyribonucleic acid synthesis. Deoxyribonucleic acid synthesis by spleen cells was measured by the incorporation of [^{3}H]thymidine as described previously (34) but with the following modifications. Spleen cells were washed twice and suspended in RPMI 1640 at a concentration of 50×10^6 cells per ml. Three 0.1-ml samples from each spleen were dispensed into separate wells of a microtiter plate (IS-FB-96-TC; Linbro Chemical Co., New Haven, Conn.). After plating, cells were collected by aspiration onto glass-fiber filters and washed with saline by using a multiple automated sample harvester (MASH-II, Microbiological Associates, Inc., Bethesda, Md.). Filters were air dried at 37°C and transferred to screw-top vials. Scintillation cocktail containing toluene, Permafluor, and Soluene (Packard Instrument Co., Downers Grove, Ill.) was added, 5 ml to each vial. Radioactivity was measured in a liquid scintillation spectrophotometer (Mark II; Nuclear-Chicago, Des Plaines, Ill.). Data are expressed as mean counts per minute of three replicate samples ± standard error of the mean.

Morphological transformation. To observe phagocytic cells, spleen cells were incubated in a dilute suspension of India ink as described previously (34), after which they were washed thoroughly in RPMI 1640 and pelleted on glass slides using a cytocentrifuge (Shandon-Elliot, Sewickley, Pa.). Slide preparations were stained by the May-Grünwald-Giemsa method and examined by light microscopy at a magnification of ×1,100. Cells were classified according to morphological criteria described previously (35).

Audioradiography. Cells were prepared for autoradiography in a manner identical to that described previously (35).

Detection of the C3 receptor by antibody and complement-coated bacteria. Quantitation of B lymphocytes by bacteria-antibody-complement (BAC) rosette formation was performed by the method of Gormus et al. (16) with the following modifications (7). *Escherichia coli* O111:K58 (Difco Laboratories, Detroit, Mich.) was grown in Trypticase soy broth by overnight incubation at 37°C. Bacteria were harvested by centrifugation (10,000 × *g* for 10 min at 4°C), washed thoroughly, and sensitized with subagglutinating amounts of specific antiserum (rabbit anti-*E. coli* O111:K58 serum; Difco, lot no. 630256; final antibody dilution, 1:100). Unreacted antibody was removed by two washes with 10-ml portions of phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA; Pentex, Miles Laboratories, Elkhart, Ind.; no. 81-013-3). The required complement components were added to the BA by suspending the cell pellet in 0.5 ml of fresh serum from C5-deficient (DBA/2) mice and incubating for 30 min at 37°C. After two 10-ml washes with PBS-BSA, 10^8 BAC were reacted with 10^6 control or LPS-activated spleen cells by incubating for 30 min at 4°C in a test tube mounted on a horizontal rotating platform revolving at 10 to 20 rpm. Spleen cells were separated from unbound bacteria by gentle centrifugation (50 × *g* for 5 min), washed twice, and resuspended in PBS-BSA at a concentration of 10^6 cells per ml. Spleen cells were deposited on microscope slides by cytocentrifugation and stained by May-Grünwald-Giemsa method or prepared for autoradiography as described above. At least 400 cells per preparation were examined, and all mononuclear cells binding four or more bacteria were scored as positive for the C3 receptor. BA prepared in the absence of complement failed to bind to control or LPS-activated spleen cells.

Fluorescent-antibody technique. Cells synthesizing immunoglobulin were quantitatively assessed by direct immunofluorescence using a fluorescein-conjugated goat anti-mouse immunoglobulin M (IgM) serum (Meloy Laboratories, Springfield, Va; lot no. 64640). Thoroughly washed spleen cells were resuspended at a concentration of 10^6 cells per ml in PBS-BSA. Cells were then deposited on glass slides by using a cytocentrifuge. Slides were air dried, fixed in methanol, and stained with fluorescein conjugates in a moist chamber for 30 min (16). Unbound conjugate was removed by washing three times with PBS. The cell pellet was overlaid in phosphate-buffered glycerol (pH 7.2), covered with a cover slip, and examined immediately with a Leitz Orthoplan fluorescence microscope equipped with phase optics and Ploem illumination. Cells synthesizing immunoglobulin were recognized by their intensely stained cytoplasm with a course granular appearance. All fluorescent staining

was inhibited by unconjugated goat anti-mouse immunoglobulin (Cappell Laboratories, Downingtown, Pa.; lot no. 8698).

Statistical evaluation. Differences in spleen cell recoveries between LPS-treated and control mice were compared by Student's t test. Values of P less than 0.025 were considered significant.

RESULTS

Gross morphological changes were observed in mouse spleens after i.v. administration of LPS. Splenomegaly occurred within 48 h after injection and was still observable for 48 to 72 h thereafter. Marked enlargement of the spleen was accompanied by a rise in nucleated spleen cells and by increased incorporation of [^{3}H]thymidine into acid-precipitable material. As little as 1 μg of LPS induced these changes; increasing the amount of LPS injected elicited correspondingly greater responses (Table 1). Cellular proliferation induced by LPS lasted approximately 5 days (Table 2). Increased [^{3}H]thymidine incorporation was detectable within 24 h of LPS administration, reached maximum values after 72 h, and declined rapidly thereafter. Enhanced cell recoveries paralleled proliferative responses throughout the 5-day period.

Differential counts of normal spleen cell suspensions demonstrated that the majority of cells were lymphocytes (Table 3); 69% were classified as small lymphocytes, while an additional 20% had identical morphology but were slightly larger. Blast cells comprised 4 to 6% of normal spleen cells. Mononuclear, phagocytic cells (macrophages) were rarely observed, and polymorphonuclear leukocytes (PML) constituted the remaining 5 to 7%. Significant alterations in the cellular composition of the spleen occurred immediately after LPS injection. Within 24 h, a two- to threefold increase in PML and blast cells was noted. The PML response was transient, returning to normal levels 48 h after injection. In contrast, the percentage of large lymphocytes and blast cells continued to increase with a reciprocal decrease in the proportion of small lymphocytes.

TABLE 1. *Effect of LPS administration on proliferation of mouse spleen cells in vivo*[a]

Treatment	Dose (μg)	Nucleated cells/spleen $\times 10^{-8}$ (mean $\pm$ SE)[b]	Incorporation of [^{3}H]thymidine (mean cpm/10^7 cells $\pm$ SE)[c]
Control	0	0.99 $\pm$ 0.10	1,648 $\pm$ 126
LPS	0.1	1.11 $\pm$ 0.09	1,851 $\pm$ 224
LPS	1	1.30 $\pm$ 0.07	7,674 $\pm$ 1,043
LPS	5	1.42 $\pm$ 0.08[d]	16,417 $\pm$ 1,910
LPS	50	1.69 $\pm$ 0.16[d]	24,710 $\pm$ 1,924
LPS	100	1.89 $\pm$ 0.17[d]	36,162 $\pm$ 5,923

[a] The indicated dose of LPS was injected i.v. at the start of this experiment. After 48 h, 25 μCi of [^{3}H]thymidine was injected i.v., and spleen cells were harvested 24 h later.

[b] Means were determined on counts from three mice. SE, Standard error.

[c] cpm, Counts per minute; SE, standard error.

[d] $P < 0.025$.

The identity of cell types proliferating in response to LPS was determined by autoradiography. In normal spleens, 3 to 4% of nucleated cells were dividing; these cells were blasts (Table 4). Only occasional small or large lymphocytes were seen to have incorporated [^{3}H]thymidine. A small percentage of PML were labeled 24 and 48 h after LPS injection. However, the greatest proportion of proliferating cells were large lymphocytes and blasts. Nearly all blasts and about one-half of large lymphocytes incorporated [^{3}H]thymidine by 72 h after injection of LPS. At this time, these cell types constituted over 50% of cells in LPS-treated spleens.

The presence of a C3 receptor on B-lymphocyte membranes was used to determine which lymphocyte subpopulations were proliferating in response to LPS. Of normal spleen cells, 27 to 43% bound BAC rosettes (data not shown). Differential counts determined that small lymphocytes and blasts were distributed equally among rosette- and non-rosette-forming cells (Table 5). Dramatic changes occurred in each lymphocyte subpopulation after injection of LPS. First, the percentage of small lymphocytes in each category decreased progressively with time. Secondly, the percentage of rosette- and non-rosette-forming blasts increased substantially. The greatest alterations appeared, however, among rosette-forming cells. After 72 h, only 13% of these cells were small lymphocytes, while 61% were blasts. At the same time, small lymphocytes and blasts comprised 65 and 20% of non-rosette-forming cells, respectively. Furthermore, a greater percentage of rosette-forming cells incorporated [^{3}H]thymidine.

In addition to blast transformation and cell division, LPS induced immunoglobulin synthesis by spleen cells in vivo. Direct staining of LPS-treated spleen cells with fluorescein-conjugated goat antisera specific for mouse immunoglobulins showed that less than 1% of normal spleen cells stained with fluorescein-conjugated antisera used in these experiments. Within 24 h after LPS injection, a substantial proportion of lymphocytes produced IgM. Cells possessing intensely fluorescent cytoplasms were first detected after 24 h, reached a maximum of 32% after 96 h, and slowly declined thereafter (Table 6). Fluorescein-conjugated antisera to mouse IgG or IgA failed to detect cells producing these immunoglobulin classes (data not shown). Morphologically, large lymphocytes and blast cells predominated among IgM-staining cells, al-

TABLE 2. *Duration of in vivo proliferative responses induced by LPS*[a]

Time after LPS injection (h)	Nucleated cells/spleen × 10^{-8} (mean ± SE)[b]		Incorporation of [^{3}H]thymidine (mean cpm/10^7 cells ± SE)[c]	
	Control	LPS	Control	LPS
24	1.09 ± 0.10	1.28 ± 0.08	2,124 ± 105	9,634 ± 958
48	1.01 ± 0.09	1.51 ± 0.15[d]	1,829 ± 412	15,172 ± 1,408
72	1.15 ± 0.09	2.00 ± 0.12[d]	2,317 ± 240	39,607 ± 4,423
96	1.08 ± 0.15	1.59 ± 0.09[d]	2,138 ± 204	19,490 ± 2,769
120	1.20 ± 0.17	1.50 ± 0.12	2,058 ± 304	6,962 ± 973

[a] Strain 7 LPS (50 μg) was administered i.v. at time zero. [^{3}H]thymidine (25 μCi) was injected i.v. at the specified times thereafter, and animals were sacrificed 24 h after this injection.
[b] Means were determined on counts from three mice. SE, Standard error.
[c] cpm, Counts per minute; SE, standard error.
[d] $P < 0.025$.

TABLE 3. *Alterations in spleen cell subpopulations after injection of LPS*[a]

Treatment	Time after injection (h)	PML[c]	Mononuclear leukocytes[b]				
			Macrophages	SL	LL	Blasts	Other
None	24	7	2	61	22	4	1
	48	3	1	72	20	6	0
	72	8	0	67	20	6	0
LPS	24	22	1	47	20	12	0
	48	6	0	37	27	29	1
	72	4	0	31	30	35	0

[a] Results are expressed as percentages and based on counts of 400 cells. LPS (50 μg) was injected i.v. at initiation. At the indicated times, spleens were harvested, and differential counts were made.
[b] All mononuclear leukocytes containing five or more phagocytic carbon particles were classified as macrophages. SL, Small lymphocytes; LL, large lymphocytes; Other, unidentifiable or degenerating cells.
[c] Includes phagocytic and nonphagocytic polymorphonuclear leukocytes.

TABLE 4. *Morphological characterization of spleen cells proliferating in response to LPS*[a]

Treatment	Time after initial injection (h)	PML[c]	Mononuclear leukocytes[b]			
			Macrophages	SL	LL	Blasts
None	24	4 (0)	1 (0)	74 (1)	18 (2)	3 (50)
	48	2 (0)	0 (0)	77 (2)	19 (3)	2 (75)
	72	4 (11)	2 (0)	65 (2)	20 (2)	9 (67)
LPS	24	19 (16)	0 (0)	56 (2)	11 (25)	14 (100)
	48	11 (0)	2 (0)	44 (5)	17 (27)	26 (80)
	72	3 (0)	1 (25)	42 (2)	24 (54)	30 (95)

[a] Results are expressed as percentages of total cells examined. C57Bl/6 mice received 50 μg of LPS i.v. Additional injections of 10 μCi of [^{3}H]thymidine were administered i.v. to control and LPS-treated mice at initiation and at 24 h thereafter. Spleens were harvested at the indicated times, and cell suspensions were prepared for cytocentrifugation and autoradiography. Slides coated with photoemulsion were exposed for 5 to 7 days. Cells possessing four or more grains directly over the nucleus were considered positive. Parentheses indicate percentage of labeled cells in each differential.
[b] Macrophages, all mononuclear, phagocytic cells; SL, small lymphocytes; LL, large lymphocytes.
[c] Includes phagocytic and nonphagocytic polymorphonuclear cells.

though small lymphocytes occasionally stained positively for IgM.

LPS extracted from several polysaccharide-deficient (rough) mutants of *S. typhimurium* also produced effects in vivo that were similar to those induced by LPS from strain 7 in vivo. Increases in nucleated spleen cell recoveries, blast transformation, and immunoglobulin-producing cells followed administration of all preparations tested (Table 7). Although some variation was obtained, significant increases or decreases in these activities could not be attributed

to the losses of O or R polysaccharides. For example, LPS from mutant TV 119, which possesses the complete R core but lacks O-specific side chains, was the most potent mitogen observed. Responses induced by LPS from SL 684 grown with or without galactose were comparable. Furthermore, LPS from SL 1102, deficient in the entire polysaccharide core and consisting only of 2-keto-3-deoxy-D-octulosonic acid and lipid A, was as active as LPS obtained from the smooth, wild-type strain 7.

DISCUSSION

These results demonstrate that, 24 h after intravenous injection of bacterial LPS into mice, striking alterations occur in the cellular composition of the spleen. Morphologically, two distinct cell types appeared to be the targets of LPS. The response of PML was transient and characterized by rapid accumulation with a relatively minor degree of cell division. In contrast, increased numbers of lymphocytes, primarily of bone marrow origin, resulted from the proliferation of lymphocytes normally residing in the spleen. These cells underwent a maturational process characterized by morphological transformation, mitosis, and immunoglobulin production.

Previous investigations have demonstrated that LPS induces splenomegaly and cellular proliferation (42, 44). Analysis of ^{32}P incorporation into ribonucleic acid and deoxyribonucleic acid and subsequent decreases in specific activity of nucleic acids indicated that spleen cells in LPS-treated mice initially divided rapidly and differentiated into mature cells with slower generation times (42). Increased [^{3}H]thymidine uptake, marked hyperplasia of B-cell zones, and greater numbers of mononuclear cells bearing surface membrane immunoglobulin have also been observed (24). Our results confirm and extend these in vivo effects of LPS. They show that the mitogenic effects of LPS on lymphoid cells in vivo parallel those effects observed previously in vitro. LPS induced a high percentage of splenic lymphocytes to transform into blast cells within 48 to 72 h after administration (Tables 3 and 4). Blast transformation was accompanied by a decrease in the percentage of spleen cells with the morphology of small lymphocytes. Observation by light microscopy indicated that the blast cells possessed decreased nuclear/cytoplasmic ratios, acentric nuclei, distinct nucleoli, clear perinuclear areas, and increased cytoplasmic basophilia. Autoradiographic analysis revealed that the majority of dividing cells were lympho-

TABLE 5. *Presence of the C3 receptor on LPS-activated spleen cells*[a]

Treatment	Time after injection (h)	Rosette-forming[b]			Non-rosette-forming		
		SL	LL	Blasts	SL	LL	Blasts
Control	24	64 (0)	33 (3)	4 (75)	87 (0)	10 (5)	3 (62)
	48	71 (0)	22 (3)	7 (90)	82 (0)	14 (7)	4 (75)
	72	70 (0)	26 (3)	4 (67)	87 (0)	12 (4)	1 (0)
LPS	24	49 (2)	24 (58)	27 (79)	83 (0)	13 (25)	4 (100)
	48	27 (2)	30 (48)	43 (76)	72 (0)	14 (17)	14 (95)
	72	13 (4)	26 (43)	61 (84)	65 (2)	15 (15)	20 (90)

[a] Differential counts were based on 200 rosette- and non-rosette-forming cells. Results are expressed as the percentage of cells within each category. Parentheses indicate percentage of labeled cells in each differential.
[b] All mononuclear cells binding five or more bacteria to surface membrane were scored as positive. SL, Small lymphocytes; LL, large lymphocytes.

TABLE 6. *Immunoglobulin synthesis after LPS administration*[a]

Treatment	Morphology[c]	Time (h) after LPS injection[b]			
		72	96	120	144
Control	SL	71 (0)	65 (0)	72 (0)	71 (1)
	LL + blasts	29 (1)	35 (0)	28 (1)	29 (0)
LPS	SL	38 (3)	36 (6)	41 (7)	52 (8)
	LL + blasts	62 (31)	64 (47)	59 (36)	48 (46)

[a] LPS (50 μg) was injected i.v. into A/J mice. At the indicated times, spleens were harvested, and cytocentrifuge slides were prepared.
[b] Differential counts on 400 cells were made; results are expressed as the percentage of total cells examined. Parentheses indicate percentage of cells staining for IgM.
[c] SL, Small lymphocytes; LL, large lymphocytes.

TABLE 7. *Characterization of the LPS moiety activating lymphocytes in vivo*[a]

Strain designation	LPS composition	Nucleated cells/spleen × 10^{-8} (mean ± SE)[b]	Incorporation of [^{3}H]-thymidine (mean cpm/10^7 cells ± SE)[c]	IgM production[d]
Uninjected controls		1.17 ± 0.05	3,239 ± 233	<1
STM 7	Heptose backbone, core, and side chains	1.91 ± 0.08	16,932 ± 1,517	25
SL 684+	Heptose backbone, core, and side chains	1.72 ± 0.13	14,474 ± 1,766	21
TV 119	Heptose backbone and core	2.26 ± 0.18	24,273 ± 2,199	19
SL 684−	Glucose and heptose backbone	2.03 ± 0.07	19,537 ± 1,993	21
SL 1102	KDO[e] and lipid A	1.67 ± 0.11	16,932 ± 1,517	16

[a] LPS (50 μg), prepared from the designated strains, was injected i.v. at time zero. [^{3}H]thymidine (25 μCi) was administered i.v. after 48 h, and spleens were harvested 24 h after this injection. Cells were counted and assayed for DNA synthesis and immunoglobulin production as described in the text.

[b] Means were determined on counts from three mice. SE, Standard error.

[c] cpm, Counts per minute; SE, standard error.

[d] Percentage of total cells staining positively for IgM.

[e] KDO, 2-Keto-3-deoxy-D-octulosonic acid.

blasts bearing complement receptors on their surface membrane (Table 5). These data suggest that dividing cells were B lymphocytes. Further evidence was provided by the demonstration that nearly half of large lymphocytes and blasts in LPS-treated spleens synthesized immunoglobulin (Table 6). These same criteria are used to characterize LPS as a nonspecific B-cell mitogen in vitro (5, 28, 30). A significant proportion of LPS-stimulated spleen cells incorporated [^{3}H]thymidine but failed to bind BAC (Table 5). The identity of receptor-negative blast cells is currently unknown. These cells could represent proliferating stem cells of myeloid, erythroid, or megakaryocytic cell lines, but are more likely of B-lymphocyte origin. Not all lymphocytes bearing surface membrane immunoglobulin possess complement receptors (7); furthermore, it has been demonstrated that some B lymphocytes lose their receptors after activation with LPS in vitro (16).

The transient accumulation and subsequent disappearance of PML appear similar to the granulocytic response commonly observed in humans and other species (22, 41). Intravenous injection of LPS produces a rapid decline in the number of PML in peripheral blood, followed by a dose-dependent granulocytosis. Granulocytopenia is thought to result from margination and sequestration. Thus, PML probably appeared in LPS-treated spleens by margination within the splenic vasculature or by egression into capillary beds; their subsequent disappearance may have been associated with the anticipated granylocytosis. Although the majority of PML were not dividing cells, a small proportion was labeled with [^{3}H]thymidine (Table 4). The relative absence of cell division was not an unexpected finding, since PML are considered highly differentiated cells. Occasional labeling could have resulted from the margination of cells that were labeled while residing in the mitotic compartment of bone marrow. LPS can induce the rapid mobilization of PML from bone marrow to the circulation, a property of LPS used to estimate the bone marrow reservoir of patients with hematological disorders (9, 13).

Several studies have indicated that LPS interaction with phagocytic cells of the monocyte-macrophage series results in morphological alterations, functional activation, and cell division (8, 12). No detectable effect of LPS on splenic macrophages was observed in these experiments, but the paucity of identifiable macrophages in spleen cell suspensions prohibited acquisition of sufficient data. However, our previous investigation, which measured LPS-induced proliferation by mononuclear cells from the normal mouse peritoneum, demonstrated a cytotoxic rather than mitogenic effect of LPS in vivo and in vitro (34).

Lymphocyte activation by LPS in vitro occurs in the absence of complement and exogenous serum factors (20, 23). Mitogenicity is believed to result from hydrophobic interactions between lipid A and surface components of B-lymphocyte

membranes. Removal of ester- and amide-linked fatty acids from LPS or lipid A drastically inhibits mitogenic activity (3, 29). Whether the diverse cellular alterations observed in vivo are also mediated through LPS interaction with cell membranes is currently unknown. Within minutes of injection, LPS associates with virtually all formed elements of peripheral blood, including lymphocytes, monocytes, granulocytes, and platelets (10, 36). With additional time, LPS accumulates in the spleen and liver and is found phagocytized by fixed cells of the reticuloendothelial system (33). Data presented here demonstrate that the loss of O and R core polysaccharides from LPS does not diminish mitogenicity in vivo, as measured by increased [^{3}H]thymidine incorporation, enhanced spleen cell recoveries, and immunoglobulin production (Table 7). Collectively, these results indicate that lipid A is essential for B-lymphocyte activation in vivo, and they support the mechanism of direct membrane interaction.

Previous studies investigating the morphological alterations occurring in lymphoid tissues after simultaneous injection of antigen and LPS revealed that enhanced antibody responses were associated with the appearance and proliferation of pyroninophilic lymphoid cells (43, 45). The intensity of lymphocyte activation and numbers of mitotic figures closely paralleled the rise and fall of serum antibodies. However, it is not possible to determine from these studies whether cellular responses were induced by LPS or protein antigen. André-Schwartz et al. observed similar morphological changes in the rabbit spleen after administration of LPS alone, but these responses could have been initiated by immunogenic polysaccharides (6). Our results show that these responses were effected through lipid A, the component of LPS responsible for adjuvant activity (11).

These studies have demonstrated several effects of LPS in vivo that could contribute to its ability to enhance antibody responses. The rapid accumulation of PML in the spleen should aid in antigen localization and retention, since antigen binds to these cells for short periods of time (26). In addition to its mitogenic effects, the capacity of LPS to induce IgM synthesis by a large proportion of B lymphocytes may also play a significant role in adjuvant activity. As products of polyclonal B-cell activation, these immunoglobulins would be expected to react with a wide array of antigenic determinants. Thus, LPS injection in vivo should result in enhanced levels of IgM antibodies in the serum, a fact previously demonstrated by Rowley and Turner (32). Whereas naturally occurring or passively administered IgG antibodies are specifically suppressive, small amounts of IgM efficiently enhance primary immune responses (18, 25).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants BRSG-77 P-4 and AI-12618 from the National Institute of Allergy and Infectious Diseases, by grants IM-138 and IN-27Q from the American Cancer Society, and by research funds from The Veterans Administration Hospital, Houston, Texas.

We thank Roberta Cooper for her excellent technical assistance and Elaine Bowers for preparation of the manuscript.

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