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Surface Phenotype of LPS-Binding Murine Lymphocytes (41821)

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Abstract. We have characterized LPS^+ murine lymphocytes by determining their surface phenotype using double-labeling immunofluorescence. In the spleen, 40% of cells were $LPS^+\mu^+$ (B cells), 5% were $LPS^+Thy1.2^+$ (T cells), and 5% were LPS^+ cells bearing neither μ nor Thy1.2 (null cells). Peripheral lymph nodes contained 11% LPS^+ B cells and 2% LPS^+ T cells, and mesenteric lymph nodes contained 15% LPS^+ B cells and 3% LPS^+ T cells. In contrast, the 4% LPS^+ cells in Peyer's patches were all B cells, and the thymus contained 4% LPS^+ T cells. Only the spleen contained LPS^+ null cells. Within each lymphoid organ examined, the fraction of total lymphocytes identified as $LPS^+\mu^+$, LPS^+Ia^+, or $LPS^+\delta^+$ was similar, indicating that LPS^+ B cells possessed the surface phenotype $\mu^+Ia^+\delta^+$, characteristic of a mature B cell. This conclusion was supported by the absence of $LPS^+\mu^+Ia^+\delta^+$ cells in newborn spleens. The fraction of $\mu^+Ia^+\delta^+$ cells which also binds LPS was highest in spleen and lowest in Peyer's patches. Assuming that cells are, on the average, more mature in the progression from spleen to lymph nodes to Peyer's patches, it would appear that LPS^+ cells are a less mature fraction of the $\mu^+Ia^+\delta^+$ pool, distinguished by the presence of an LPS binding site or receptor. These data illustrate selective binding of LPS predominantly to mature B cells, but also to small numbers of null cells and T cells. The relationship of this binding to cell activation is discussed by considering the characteristics of cells which can be activated by LPS to clonal growth or differentiation under appropriate conditions.

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is a complex macromolecule both structurally and functionally. Of its three regions, the serologically detected O antigen, core, and lipid A, the latter bears primary responsibility for most biological effects including metabolic and cellular changes, fever, hypotension and intravascular coagulation which can lead to death (1, 2). Within the lymphoid system, the major lymphocyte targets for LPS are bone marrow-derived lymphocytes (B cells) which are activated to DNA synthesis and immunoglobulin synthesis and secretion without the necessity for T-cell help (3). The singular preference of LPS for B cells would suggest these cells have a specific binding site or receptor for LPS.

B-cell receptors for mitogens or polyclonal B-cell activators have been discussed at length by Möller (4) and Coutinho (5) in considering mechanisms of B-cell triggering. Although only scant physical evidence has been provided for such receptors (6), the terminology has remained in use, often as shorthand to describe a structure whose presence is dictated by the exigency of a model (7, 8). Evidence for such a site has been sought in investigations designed to determine if LPS binds preferentially to subpopulations of lymphocytes. Some studies have reported increased uptake of radiolabeled LPS or lipid A by peripheral B cells or lymphocytes from normal or nude mice compared to thymocytes or T cells (9–11), but no differences were detected in other experiments (9, 12, 13). Differences were observed either when purified lymphocyte populations or low doses (1–50 μg/ml) of LPS were used. However, in all studies, cells were incubated with LPS at 37°C for several hours or days. Since cyclical fluctuations in LPS binding may occur at 37°C (14) and events subsequent to binding such as pinocytosis or capping can change the amount of LPS which is detectable at any given time, quantitative estimates of LPS uptake in these systems may not be limited to the initial binding.

In contrast, preferential binding of LPS and lipid A to B cells has been demonstrated on an individual cell basis using radioautography.

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Bona *et al.* (9) found 17% of thymus cells and 47% of splenic lymphocytes were LPS^+. Gregory *et al.* (15) found that no T cells and 22–59% of Ig^+ spleen cells bound lipid A, depending on the strain examined. Furthermore, in both studies the number of LPS^+ cells was given for only one concentration of ligand, raising some question as to the significance of the actual numbers of LPS^+ cells detected. Finally, incubations were carried out at room temperature or 37°C in the absence of azide, leaving open the possibility that postbinding events influenced the number of LPS^+ cells seen.

In the experiments reported here, we employed techniques designed to avoid the problems inherent in previous studies. We used a sensitive hapten-sandwich immunofluorescence technique to detect individual cells which bind low concentrations of LPS and carried out incubations at 0°C in the presence of azide to prevent capping and other temperature-dependent events which might change the localization of bound ligand. We used a hapten-sandwich immunofluorescence procedure to provide a higher degree of sensitivity than can be achieved with conventional indirect immunofluorescence. In addition, we have further characterized LPS^+ lymphocytes by evaluating the presence of other surface markers using double-labeling immunofluorescence. We found that the predominant LPS^+ cell in the spleen is μ^+, but a small fraction of $Thy1.2^+$ cells also binds LPS. The largest number of LPS^+ T cells were found in the spleen whereas none (<0.2%) were found in Peyer's patches. On the basis of the number and distribution of cells bearing μ, Ia, and δ, and the fraction of these which are LPS^+, we concluded that LPS binds predominantly to mature B cells with the surface phenotype $\mu^+Ia^+\delta^+$. This conclusion was verified by the virtual absence of $LPS^+\mu^+Ia^+\delta^+$ cells in newborn spleens.

Materials and Methods. *Animals.* Male and female New Zealand white rabbits were obtained from Beckens Research Animal Farms, Sanborne, N.Y. Adult female and pregnant C3H/St were purchased from the West Seneca Laboratory of Health Research Inc., West Seneca, New York, and female C3H/HeJ mice from the Jackson Laboratories, Bar Harbor, Maine. Adult mice were used as cell donors at 8 to 14 weeks of age and newborns at 1 and 6 days of age. All animals were maintained in the animal facilities at the State University of New York at Buffalo and given food and water *ad libitum.*

Lipopolysaccharide (LPS). Purified LPS for binding studies was prepared from lyophilized *Escherichia coli* 055:B5 (New England Enzyme Center, Boston, Mass.) by extraction with phenol–water, enzyme treatment, and column chromatography (16). LPS solutions were sterilized by passage through a 0.45-μm filter, stored at 4°C at 40–50 mg/ml, and diluted just before use. Nonlyophilized purified LPS was used in these studies as lyophilized material gave more variable results. LPS concentration was determined by the thiobarbituric acid assay for deoxyhexoses (17) using weighed lyophilized LPS of the same strain as a standard. LPS preparations contained <2.0% protein detectable by the Lowry modification of the Folin–Ciocalteau procedure (18) and did not stimulate mitogenesis in C3H/HeJ spleen cells at a concentration of 100 μg/ml. Commercial preparations of phenol-extracted LPS used in hemagglutination assays and inhibition studies were obtained as follows: from *Salmonella typhimurium,* Sigma, St. Louis, Missouri; from *E. coli* strains 0111:B4 and 0127:B8, Difco, Detroit, Michigan.

Anti-LPS and anti-hapten antisera. Antiserum against the O-specific antigen of *E. coli* 055:B5 was produced by immunization of rabbits with three weekly intravenous injections of 0.2 ml of a 0.2 mg/ml suspension of dried bacteria in saline. Sera were tested at monthly intervals for agglutinating antibody against sheep red blood cells coated with LPS (19). After 5 months of immunization, the antisera contained 2-mercaptoethanol (2-ME) (MCB, Norwood, Ohio)-resistant antibodies against 055:B5 LPS at titers >10,240, while titers against LPS from *E. coli* 0111:B4 and 0127:B8 were <4. Anti-hapten antisera were prepared in rabbits immunized by standard procedures with keyhole limpet hemocyanin (KLH; Calbiochem-Behring Corp., La Jolla, Calif.) conjugated with the diazo derivatives of *p*-azophenylarsonate (ars; Matheson, Coleman and Bell, East Rutherford, N.J.), *p*-aminophenyl β-D-lactoside (lac; Calbiochem, La Jolla, Calif.), and *p*-aminophenyl glycine (gly; ICN Pharmaceuticals, Inc., Plainview, N.J.).

Hybridoma antibodies. Hybridoma line 10-3.6 (20) secretes a γ_{2a} antibody which binds to the gene product (Ia-1) of the I-A subregion of the H-2 locus of mice bearing the k, f, r, and s haplotypes. The subclone (10-3.6-27) producing this anti-Ia was a generous gift from Dr. Philippa Marrack and Dr. John Kappler, National Jewish Hospital, Denver, Colorado, and was grown in BALB/c mice which had been pretreated with 0.5 ml pristane (2,4,6-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, Wisc.). Ascites fluid was harvested and stored at −20°C.

Hybridoma line 10-4.22 (20) was obtained from the Salk Institute Cell Distribution Center, La Jolla, Calif., and was used without subcloning. This line secretes a γ_{2a} anti-Ig-5^a (anti-δ) which binds to the heavy chain of IgD from a, c, d, and f allotype mice. Antibodies were obtained from the culture fluid of the cell line which was grown in RPMI-1640 containing glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (FBS) (medium constituents from Gibco, Grand Island, N.Y.), and 0.05 μM 2-ME. Culture supernates were harvested and frozen at −70°C until use.

Hapten modification of immunoglobulins and immunoglobulin fragments. IgG fractions were prepared from rabbit anti-hapten antisera, ascites fluid containing anti-Ia and culture supernatant fluid containing anti-δ by ammonium sulfate precipitation and chromatography on DEAE-cellulose (DE-32, Whatman Ltd., Kent, England) by standard procedures (21, 22). Fab fragments of some IgG fractions were prepared by papain digestion as described by Cammisuli and Wofsy (22). Ars–anti-LPS, gly–anti-δ, and lac–Fab–anti-Ia were prepared from the diazonium derivatives of ars, gly, and lac using the bifunctional amidating reagent methyl hydroxybenzimidate (HB) (23). HB was synthesized as described by Cammisuli and Wofsy (21). Fab fragments of anti-Ia were used for conjugation with lac as this hapten decreases the solubility of IgG to which it is conjugated (22). Haptenated reagents were stored lyophilized at 4°C.

Fluorescein and rhodamine-conjugated immunoglobulins. Rhodamine B isothiocyanate (RBITC; BDH Biochemicals LTD., Poole, England) conjugates of the IgG fractions of rabbit anti-ars, anti-lac, and anti-gly were prepared by standard procedures (24). Fluorescein conjugates of anti-lac and anti-gly were prepared following the same procedure except that fluorescein isothiocyanate (FITC; Isomer I, Sigma, St. Louis, Mo.) was added directly to the solution of IgG at 10 μg/mg IgG and stirred to become evenly suspended. Conjugates with an OD ratio of 2.0–3.0 (corresponding to a molar F/P ratio of 1.0–1.5 (25)) were dialyzed against water overnight and lyophilized for storage.

Additional immunofluorescence reagents. Goat anti-μ and RBITC–rabbit anti-goat IgG were obtained from Cappel Laboratories Inc., Cochranville, Pennsylvania. FITC–rabbit anti-goat IgG was purchased from Gibco, Grand Island, N.Y. Biotin–anti-Thy 1.2 (monoclonal antibody, clone 30-H12) and FITC–avidin were from the Becton Dickinson Monoclonal Antibody Center, Sunnyvale, California. Commercial reagents were stored according to the directions of the supplier.

Cell suspensions. Mice were sacrificed by cervical dislocation, lymphoid organs were removed, and cell suspensions prepared by standard procedures (25). Spleen cells used for immunofluorescence studies were subjected to separation on Ficoll–Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.) to remove the majority of erythrocytes, platelets, granulocytes, macrophages, and nonviable cells. Lymphocytes were >95% viable by trypan-blue exclusion. Cells used for immunofluorescence were resuspended in balanced salt solution (BSS) (25) containing 0.2% NaN_3 unless otherwise indicated, and those used for culture were resuspended in RPMI 1640 (see below).

Immunofluorescence. Immunoglobulin reagents used for immunofluorescence were dissolved in veronal buffered saline (VBS: 1 g/liter sodium barbital, 8.3 g/liter NaCl, 0.2% NaN_3, pH 7.3) with 5% fetal bovine serum (FBS) and stored as aliquots at −40°C. Reagents were thawed only once and cleared of aggregates by centrifugation in a microfuge (Beckman Instruments Inc., Palo Alto, Calif.) for 5 min prior to use. In preliminary experiments, a dose-dependent increase in the number of LPS^+ cells was obtained in all cell populations except thymus over a 2- to 30-μg/ml dose range, and no increase in this

number was seen up to 100 μg/ml. Maximum binding was achieved by 30 min of incubation, and no differences in LPS^+ cells were detected in incubations carried out at 0°C, room temperature or 37°C. Therefore cells were incubated in 2 ml BSS containing 5×10^6 cells and 30 μg/ml LPS at 0°C for 30 min unless otherwise indicated. The cells were then washed twice with VBS–FBS, resuspended in 50 μl each of ars–anti-LPS, and the haptenated antibody to the individual cell surface marker, and incubated for 20 min at 0°C. After a further two washings in VBS–FBS, cells were resuspended in 50 μl each of RIBTC–anti-ars and the FITC–labeled antibody to the appropriate hapten and incubated under the same conditions. After washing, the cells were fixed in 1% paraformaldehyde, mounted, and examined using a Leitz Dialux microscope equipped for epi-illumination with a Ploempak 2.4, filter system I (for fluorescein), filter system N (for rhodamine) and a mercury lamp. (E. Leitz, Rockleigh, N.J.). A minimum of 300 lymphocytes per slide were counted. All antibodies were titrated and used at optimal dilutions. No background staining was detected for Ia, δ or Thy1.2. Background staining for μ and LPS varied from 0 to 2% total lymphocytes. In each experiment, the surface phenotype of B-cell populations was determined using RBITC–anti-μ; and δ and Ia were detected with the appropriate haptenated antibodies and FITC-labeled anti-hapten reagents. For simplicity the data identifying separate subpopulations of LPS^+ lymphocytes have been divided into T and B cells for presentation, although individual experiments included all combinations of LPS and surface markers.

Mitogen assay. Cells were cultured in RPMI-1640 (GIBCO, Grand Island, N.Y.) containing 0.05 μM 2-ME and assayed for DNA synthesis by pulsing with 0.5 μCi/well ^{125}IUdR (Amersham/Searle, Arlington Heights, Ill.) as previously described (26).

Results. *LPS-binding splenic lymphocytes.* Cells in the spleen which are positive for membrane bound LPS (LPS^+) exhibited a characteristic fine punctate staining pattern. Brightly stained large cells with the morphology of macrophages were occasionally seen but were not included in the counts of either total cells or stained cells. The possible contribution of the Fc receptor to the detection of LPS^+ cells was examined using Fab fragments of ars–anti-LPS and RBITC–anti-ars. No difference was seen in the number of LPS^+ cells detected by fragments or intact reagents. (Data not shown.)

Forty percent of the splenic lymphocytes from both C3H/St and C3H/HeJ (LPS-hyporesponsive) mice were identified as LPS^+ B cells by the presence of bound LPS and surface μ (Table I). The B-cell population as defined by surface μ represents 53% of the splenic lymphocytes and accounts for 80% of the LPS^+ cells. Within the B-cell population, 75% μ^+ cells bind LPS while 92% of δ^+ and Ia^+ lymphocytes are LPS^+. Although some T cells have surface Ia determinants, a very low number of μ^-Ia^+ cells were detected here (<2% lymphocytes), so in this system Ia is essentially a B-cell marker. The distribution of LPS^+ cells in both high- and low-LPS responder strains is identical.

LPS-binding B cells in other lymphoid organs. Only 11% of the lymphocytes in peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) are LPS^+ cells. In comparison to splenic cells, only 40% μ^+ and 61% of δ^+ and Ia^+ cells in PLN and slightly higher fractions of MLN cells with the two latter markers are LPS^+ (Table II). The number of LPS^+ cells, the fraction of B cells which are LPS^+, and the fraction bearing Ia and δ is smaller in the Peyer's patches (PP) than in lymph nodes.

These data indicate that LPS^+ cells are predominantly B cells and LPS^+ B cells represent a subpopulation of these lymphocytes. We observed that the fraction of total lymphocytes which bear LPS *and* either μ, Ia, or δ, was essentially the same within each cell population: 39–44% in spleen, 10–12% in PLN, 14–18% in MLN, and 4–5% in PP. This suggested that the LPS^+ B cell has the surface phenotype $\mu^+Ia^+\delta^+$, and that it is the same cell type which binds LPS in all the lymphoid organs examined.

LPS-binding T cells. The number of $LPS^+Thy1.2^+$ cells was determined in the same experiments in which B-cell surface markers were examined. The results obtained with cells from the same sources as well as from thymus are seen in Fig. 1. In all populations examined except PP, a small but significant percentage

TABLE I. SURFACE PHENOTYPE OF LPS^+ C3H/St AND C3H/HeJ SPLENIC LYMPHOCYTES

	C3H/St		C3H/HeJ	
Marker	% Lymphocytes	% LPS^+ [a]	% Lymphocytes	% LPS^+
LPS^+	51.3 ± 3.6 [b]	100	49.8	100
μ^+	52.6 ± 2.3		52.2	
$LPS^+\mu^+$	39.2 ± 1.7	75.1 ± 0.8	39.9	76.5
δ^+	44.8 ± 2.7		46.2	
$LPS^+\delta^+$	41.2 ± 2.0	92.1 ± 1.1	41.9	90.8
Ia^+	47.5 ± 5.8		48.8	
LPS^+Ia^+	43.6 ± 4.2	91.7 ± 3.5	45.3	92.8
$\mu^+\delta^+$	45.6 ± 1.6	N.A. [c]	N.D. [d]	N.A.
μ^+Ia^+	48.6 ± 3.7	N.A.	N.D.	N.A.
Thy1.2$^+$	22.3 ± 5.3		24.4	
LPS^+Thy1.2$^+$	5.3 ± 1.4	23.7 ± 4.6	5.4	22.2

[a] Percentage of cells with a given marker which also binds LPS.
[b] Mean determination from three to four experiments ± SD. One experiment for C3H/HeJ.
[c] N.A., not applicable.
[d] N.D., not done.

of T cells bound LPS. These LPS^+ T cells represent 10% of spleen, 15% of MLN, 15% of PLN, and 100% of thymus lymphocytes binding LPS. Although the spleen contains fewer T cells than MLN, PLN, PP, and thymus, they are the richest source of LPS^+ T cells.

LPS-binding null cells. LPS^+ cells from the various sources tested were found to include the following proportions of B and T cells: spleen—80% B/10% T; PLN—85% B/15% T; MLN—85% B/15% T; PP—100% B; thymus—100% T. Thus up to 10% LPS^+ spleen cells appear to have neither Thy1.2 nor μ on their surface. Since 50% of spleen cells are LPS^+, 5% of splenic lymphocytes are LPS^+ null cells. Detectable numbers of LPS^+ null cells do not appear in lymphoid organs other than spleen.

LPS^+ cells and LPS mitogen responses in

TABLE II. PHENOTYPE OF LPS^+ CELLS IN LYMPHOID ORGANS

	Peripheral lymph nodes		Mesenteric lymph nodes		Peyer's patches	
Marker	% Lymphocytes	% LPS^+ [a]	% Lymphocytes	% LPS^+	% Lymphocytes	% LPS^+
LPS^+	12.2 ± 2.6 [b]	100	17.6 ± 2.1	100	6.0 ± 1.1	100
μ^+	26.1 ± 2.5		33.4 ± 2.7		35.5 ± 4.8	
$LPS^+\mu^+$	10.6 ± 3.1	40.4 ± 6.0	14.6 ± 3.0	40.2 ± 10.0	4.8 ± 0.2	13.2 ± 1.3
δ^+	18.7 ± 1.6		22.5 ± 0.8		8.4 ± 2.3	
$LPS^+\delta^+$	11.6 ± 3.1	61.7 ± 11.8	15.2 ± 0.2	67.5 ± 1.9	4.4 ± 1.0	56.7 ± 27.9
Ia^+	19.6 ± 1.6		24.0 ± 2.4		16.7	
LPS^+Ia^+	11.9 ± 2.3	61.1 ± 17.0	17.6 ± 1.5	73.0 ± 1.0	4.8	28.6
$\mu^+\delta^+$	18.7 ± 1.6	N.A. [c]	23.8 ± 1.2	N.A.	11.7	N.A.
μ^+Ia^+	19.2 ± 0.5	N.A.	23.8 ± 0.8	N.A.	17.8	N.A.

[a] Percentage of cells with a given marker which also bind LPS.
[b] Mean determination from three to four experiments ± SD.
[c] N.A., not applicable.

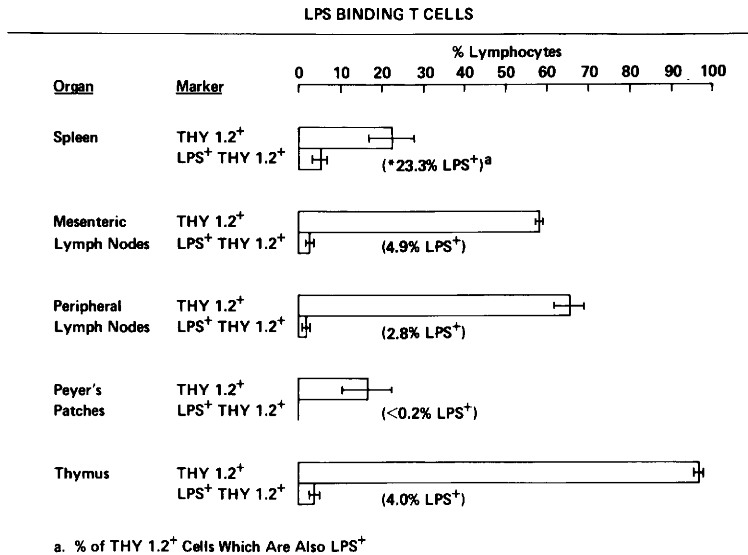

FIG. 1. LPS-binding T cells. Data taken from same experiments presented in Tables I and II.

neonatal spleen. The observation that LPS^+ cells have the surface phenotype of a mature B cell suggested that the ability to bind LPS is acquired at an identifiable stage in the ontogenetic development of this cell type. To test this in a more rigorous manner we determined the number and surface phenotype of LPS binding splenic B cells in 1- and 6-day-old mice (Table III). At 1 day postbirth, the spleen contains approximately 4% μ^+ cells, a few Ia^+ cells, no detectable δ^+ cells, and 1.5% $Thy1.2^+$ cells. Virtually all the LPS^+ cells (7.5%) in 1-day-old spleens are null cells. In the spleen of 6-day-old mice, cells bearing δ have appeared, and the number of cells exhibiting other B-cell surface markers has noticeably increased. LPS^+ cells have increased in number, but the majority are still null cells. However, a small percentage of total cells (1.2–1.4%) also bear μ, Ia, or δ in addition to LPS. Thus, in the 6-day-old spleen, as in the adult spleen (Table I), the LPS^+ B cells are confined to and represent a sizeable proportion of the $\mu^+Ia^+\delta^+$ subpopulation.

To determine whether LPS responsiveness is acquired at the same time in B-cell maturation as the capacity to bind detectable LPS, we used a portion from each of the cell pools analyzed to perform a parallel assay for LPS induced mitogenesis (Fig. 2). Cells isolated from 1-day spleen exhibit only a small amount

TABLE III. LPS-BINDING CELLS IN NEWBORN SPLEEN

Age	Marker	Percentage lymphocytes	Percentage $LPS^{+\,a}$
1 Day[b]	LPS^+	7.4[c]	100
	μ^+	3.8	
	$LPS^+\mu^+$	0.2	6.4
	δ^+	<0.1	
	$LPS^+\delta^+$	<0.1	0.0
	Ia^+	0.4	
	LPS^+Ia^+	0.1	16.7
	$Thy1.2^+$	1.5	
	$LPS^+Thy1.2^+$	<0.08	0.0
6 Day[b]	LPS^+	12.7	100
	μ^+	7.4	
	$LPS^+\mu^+$	1.2	15.9
	δ^+	1.4	
	$LPS^+\delta^+$	1.2	83.3
	Ia^+	2.1	
	LPS^+Ia^+	1.4	65.5
	$Thy1.2^+$	2.9	
	$LPS^+Thy1.2^+$	0.08	2.7

[a] Percentage of cells with a given marker which also bind LPS.

[b] Cell suspensions were prepared from pooled tissue of 20–25 animals and incubated with LPS at 30 μg/ml for 30 min at 0°C. Spleen cells from adult animals were examined in the same experiment, and the percentage of cells bearing the given markers were within 10% of the values for C3H/St given in Table I.

[c] There were 1200–1350 cells examined for each surface marker.

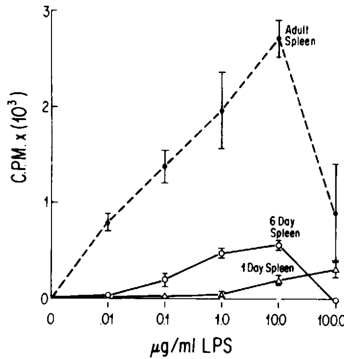

FIG. 2. LPS induced mitogenic responses in spleen cells from one-day-old, 6-day-old, and adult C3H/St mice. Assays were as previously described (15). Cultures were pulsed with 0.5 μCi [^{125}I]UdR for the last 18 hr of a 48-hr incubation period before harvesting. Results are given as mean cpm ± SD of quadruplicate cultures.

of DNA synthesis at a dose which is uncharacteristically high compared to an adult mitogenic response. In contrast to the 1-day cells, 6-day splenic cells produce a mitogenic dose–response curve which appears to differ only in magnitude from that of adult cells.

Discussion. We have used double-labeling immunofluorescence microscopy to characterize the surface markers of LPS-binding lymphocytes in spleen, lymph nodes, Peyer's patches, and thymus. The majority of LPS$^+$ cells in each organ examined except the thymus are B cells bearing the surface phenotype $\mu^+Ia^+\delta^+$. However, small numbers of T cells in all organs except PP bind LPS.

Studies using neonatal animals and induced phenotypic conversion have established that murine B-cell ontogeny is reflected in the sequential expression of surface μ, Ia, and δ (27–29). There are thus three cell populations characterized by the presence of these surface markers μ^+, μ^+Ia^+, and $\mu^+Ia^+\delta^+$. In all lymphoid tissues examined, the same numbers of LPS$^+\mu^+$, LPS$^+$Ia$^+$, and LPS$^+\delta^+$ cells are detected within each lymphoid organ, suggesting that LPS$^+$ cells are within the most mature population, $\mu^+Ia^+\delta^+$.

The results obtained with neonatal spleen cells show that LPS-binding B cells are not found until 6 days after birth when these cells have begun to acquire surface δ. Parallel examination of LPS-stimulated mitogenesis in cell populations from these newborns confirms that the acquisition of significant sensitivity to LPS as a mitogenic trigger is coincident with the appearance of the LPS binding $\mu^+Ia^+\delta^+$ lymphocyte. Thus, while LPS binding and responsive B cells are not exclusively confined to the subpopulation that exhibits this surface phenotype, we conclude that this cell type represents the majority of LPS binding and responsive B cells.

A comparison of the fraction of $\mu^+Ia^+\delta^+$ cells binding LPS in the different lymphoid organs is of further interest. As seen in Table IV, this fraction decreases on moving from spleen through lymph nodes to Peyer's patches. Since it is commonly accepted that, on the average, cells in Peyer's patches are more mature than those in the lymph nodes which are in turn more mature than those in the spleen, the observed diminution in the fraction of $\mu^+Ia^+\delta^+$ B cells which are LPS$^+$ suggests that these are a less mature subpopulation of cells of this phenotype.

The phenotype we have described appears also to be that of the B cell which is activated by LPS. Pretreatment of splenic lymphocytes with anti-Ia serum and complement has been found to abolish the subsequent proliferative response to LPS (30). The inclusion of antibodies to μ, δ, or Ia in cultures of spleen cells has been reported to inhibit LPS stimulated mitogenesis and immunoglobulin secretion (30–32). Utilizing a cell-sorter, Gronowicz *et al.* (33) prepared spleen cells which had been both positively and negatively selected for surface μ, δ, and γ and determined the polyclonal response of each population after 1 to 5 days of culture. They demonstrated that cells pos-

TABLE IV. LPS-BINDING $\mu^+Ia^+\delta^+$ B CELLS

Cell source	Percentage lymphocytes[a]	Percentage LPS$^+$[b]
Spleen	45.6	90.4
Mesenteric lymph nodes	23.8	63.9
Peripheral lymph nodes	18.7	62.0
Peyer's patches	11.7	34.5

[a] The percentage of $\mu^+Ia^+\delta^+$ lymphocytes within each cell source are taken as the percentage of lymphocytes which are $\mu^+\delta^+$.

[b] The percentage of $\mu^+Ia^+\delta^+$ which also bind LPS (taken as LPS$^+\delta^+$). Calculations based on data from Tables I and II.

itively selected for μ or δ or negatively selected to lack γ gave good responses to LPS while the responses of populations lacking either μ or δ were up to 20-fold lower. Our binding data is thus consistent with the functional data which identifies the surface phenotype of LPS-responsive cells, suggesting that the binding we detect is correlated with lymphocyte activation as assessed by LPS-induced proliferation.

Although 75% splenic B cells are LPS$^+$ (Table I), only a portion of these cells may be directly responsive to LPS. The limiting dilution analysis of Andersson *et al.* (34) estimated that only one in three splenic B cells could be induced to Ig secretion by LPS. This estimate was confirmed by Wetzel and Kettman (35) who found that in single cell cultures 25% of splenic B cells were activated to clonal growth by LPS. These investigators also reported that dextran sulfate, although capable of stimulating growth in only 5% of B cells, when added with LPS resulted in synergy in which 80% of B cells were activated. The authors concluded that the combination of dextran sulfate and LPS removed the requirement for cell interaction in growth induction. The results of these two studies suggest that all the LPS$^+$ splenic B cells we detect can be activated by LPS but that some have additional triggering requirements. These LPS$^+$ but nonresponsive splenic B cells may be analogous to those found in PP which do not give a mitogenic response to LPS in the absence of soluble factors or macrophages (36). LPS$^+$ cells with different triggering requirements may also be found in lymph nodes.

Gregory *et al.* (15) found that 22–23% of the Ig$^+$ cells from both high- and low-responder C3H strains bound lipid A, which is close to the fraction of B cells induced to Ig secretion (34) but only one-third of the number of LPS$^+$ cells we have found. This discrepancy might reflect quantitative differences in LPS binding, a point which is currently under investigation in our laboratory. No data were reported on other B-cell surface markers or the organ distribution of LPS$^+$ cells. They also concluded that 2–3% of LPS$^+$ cells were null cells and that no θ^+ cells bound lipid A. However, these authors assumed that lipid A binding to thymocytes was nonspecific and set their background accordingly thus precluding detection of LPS$^+$ T cells. The recent report that small numbers of normal T cells proliferate in response to LPS (37) is consistent with our demonstration of LPS$^+$ T cells.

LPS$^+$ null cells detected in adult and neonatal spleen may include immature cells which are induced by LPS *in vitro* to express the characteristic antigens of mature T cells (Thy-1) or mature B cells (μ, Ia, and δ) (27, 32, 38, 39). Some cells are presumably the normal equivalent of pre-B-cell lines which respond to LPS by expression of cytoplasmic (40) or membrane immunoglobulin (41). The existence of a B-cell lymphoma possessing μ as the only surface immunoglobulin, which responds to LPS by increased secretion of IgM (42), suggests that LPS$^+\mu$-only cells exist in normal mice in very small numbers which might not be detected in our assay.

Our observation that identical numbers of LPS$^+$ cells are present in C3H/St and C3H/HeJ spleens are in keeping with the majority of published observations. No difference in binding to spleen cell populations of normal and hyporesponsive strains at any concentration of radioactive LPS or lipid A has been detected either in mass cultures or at the single cell level (10, 11, 13, 15, 43, 44). In contrast, Nygren *et al.* (45) detected fewer LPS-binding spleen cells in hyporesponsive than in normal mice using immunoelectronmicroscopy. Although Coutinho and his colleagues reported an anti-LPS-receptor antiserum which reacted with normal but not hyporesponsive spleen cells (6), attempts to reproduce this finding have not been successful ((46) and Jacobs, unpublished). Thus the genetic defect in the C3H/HeJ strain would appear not to reflect the loss of high-affinity lymphocyte binding sites for LPS, and is more likely to be due to a defect in a postbinding activation step localized in the membrane (47).

The demonstration that all lymphocytes do not bind LPS when exposed to low doses of the ligand indicates that LPS binds selectively to subpopulations of lymphocytes. The populations include most mature B cells and small numbers of peripheral T cells and null cells. The number of LPS$^+$ cells corresponds to the proportion of these cells found by other investigators to be activated by LPS to prolif-

eration or differentiation. The structural specificity of the binding site and its role in LPS activation of cells remain to be determined.

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1. Morrison DC, Ryan JL. Bacterial endotoxins and host immune responses. In: Dixon FG, Kunkel HG, eds. Advances in Immunology. New York, Academic Press, Vol 28:pp293–450, 1979.
2. Morrison DC, Ulevitch RJ. The effects of bacterial endotoxins on host mediation systems. Amer J Pathol **93**:527–617, 1978.
3. Gery I, Kruger J, Spiesel SZ. Stimulation of B-lymphocytes by endotoxin: Reactions of thymus-deprived mice and karyotypic analysis of dividing cells in mice bearing T_6T_6 thymus grafts. J Immunol **108**:1088–1091, 1972.
4. Möller G. One nonspecific signal triggers B lymphocytes. Transplant Rev **23**:126–137, 1975.
5. Coutinho A. The theory of the "one nonspecific signal" model for B cell activation. Transplant Rev **23**:49–65, 1975.
6. Forni L, Coutinho A. An antiserum which recognizes lipopolysaccharide-reactive B cells in the mouse. Eur J Immunol **8**:56–62, 1978.
7. Andersson J, Coutinho A, Lernhardt W, Melchers F. B-Cell Triggering. In: Cooper M, Mosier DE, Scher I, Vitetta ES, eds. B Lymphocytes in the Immune Response. New York, Elsevier/North-Holland, pp257–262, 1979.
8. Rosenspire AJ, Rosen R, Jacobs DM. A general interactive model for B cell activation. I. The theory. Cell Biophys **3**:71–87, 1981.
9. Bona C, Juy D, Truffa-Bachi P, Kaplan GJ. Binding, capping and internalization of lipopolysaccharide in thymic and non-thymic lymphocytes of the mouse. Biological and autoradiographic study. J Microsc Biol Cell **25**:47–56, 1976.
10. Kabir S, Rosenstreich DL. Binding of bacterial endotoxin to murine spleen lymphocytes. Infect Immun **15**:156–164, 1977.
11. Zimmerman DH, Gregory S, Kern M. Differentiation of lymphoid cells: The preferential binding of the lipid A moiety of lipopolysaccharide to B lymphocyte populations. J Immunol **119**:1018–1023, 1977.
12. Möller G, Andersson J, Pohlit H, Sjöberg O. Quantitation of the number of mitogen molecules activating DNA synthesis in T and B lymphocytes. Clin Exp Immunol **13**:89–99, 1972.
13. Symons DBA, Clarkson CA. The binding of LPS to the lymphocyte surface. Immunology **38**:503–508, 1979.
14. Davies M, Stewart-Tull DES, Jackson DM. The binding of lipopolysaccharide from *Escherichia coli* to mammalian cell membranes and its effect on liposomes. Biochim Biophys Acta **508**:260–276, 1978.
15. Gregory SH, Zimmerman DH, Kern M. The lipid A moiety of lipopolysaccharide is specifically bound to B cell subpopulations of responder and nonresponder animals. J Immunol **125**:102–107, 1980.
16. Morrison DC, Leive L. Fractions of lipopolysaccharide from *Escherichia coli* 0111:B4 prepared by two extraction procedures. J Biol Chem **250**:2911–2919, 1975.
17. Cynkin MA, Ashwell G. Estimation of 3-deoxy sugars by means of the malonadehyde–thiobarbituric acid reaction. Nature (London) **186**:155–156, 1960.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
19. Neter E. Bacterial hemagglutination and hemolysis. Bacteriol Rev **20**:166–188, 1956.
20. Oi VT, Jones PP, Goding JW, Herzenberg LA, Herzenberg LA. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. In: Melchers F, Potter M, Warner N, eds. Lymphocyte Hybridomas. Current Topics in Microbiology and Immunology. New York, Springer-Verlag, Vol 81:pp115–129, 1978.
21. Cammisuli S, Wofsy L. Hapten-sandwich labeling. III. Bifunctional reagents for immunospecific labeling of cell surface antigens. J Immunol **117**:1695–1704, 1976.
22. Garvey JS, Cremer NC, Sussdorf DH. Methods in Immunology. Reading, Mass., Benjamin, pp267–270, 1977.
23. Wallace EF, Wofsy L. Hapten-sandwich labeling. IV. Improved procedures and non-cross-reacting hapten reagents for double-labeling cell surface antigens. J Immunol Methods **25**:283–289, 1979.
24. Brandtzaeg P. Conjugation of immunoglobulin G with different fluorochromes. I. Characterization by anion-exchange chromatography. Scand J Immunol **2**:273–290, 1973.
25. Mishell RI, Dutton RW. Immunization of dissociated spleen cell cultures from normal mice. J Exp Med **126**:423–442, 1967.
26. Jacobs DM, Morrison DC. Dissociation between mitogenicity and immunogenicity of TNP-lipopolysaccharide (TNP-LPS), a T-independent antigen. J Exp Med **141**:1453–1458, 1975.
27. Hämmerling U, Chin AF, Abbott J. Ontogeny of murine B lymphocytes: Sequence of B cell differentiation from surface-immunoglobulin-negative precursors to plasma cells. Proc Natl Acad Sci USA **73**:2008–2012, 1976.
28. Abney ER, Cooper MD, Kearney JF, Lawton AR, Parkhouse RME. Sequential expression of immunoglobulin on developing mouse B lymphocytes: A

systematic survey that suggests a model for the generation of immunoglobulin isotype diversity. J Immunol **120**:2041–2049, 1978.
29. Kearney JF, Cooper MD, Klein J, Abney ER, Parkhouse RME, Lawton AR. Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. J Exp Med **146**:297–301, 1977.
30. Niederhuber JE, Frelinger JA, Dugan E, Coutinho A, Schreffler DC. Effects of anti-Ia serum on mitogenic responses. I. Inhibition of the proliferative response to B cell mitogen, LPS, by specific anti-Ia sera. J Immunol **115**:1672–1676, 1975.
31. Kincade PW, Ralph P. Regulation of clonal B-lymphocyte proliferation by anti-immunoglobulin or anti-Ia antibodies. Cold Spring Harbor Symp Quant Biol **41**:245–250, 1976.
32. Sitia R, Abbott J, Hämmerling U. The ontogeny of B lymphocytes. V. Lipopolysaccharide-induced changes of IgD expression on murine B lymphocytes. Eur J Immunol **9**:859–864, 1979.
33. Gronowicz ES, Doss C, Assisi F, Vitetta ES, Coffman RL, Strober S. Surface isotypes on cells responding to lipopolysaccharide by IgM and IgG secretion. J. Immunol **123**:2049–2056, 1979.
34. Andersson J, Coutinho A, Lernhardt W, Melchers F. Clonal growth and maturation to immunoglobulin secretion *in vitro* of every growth-inducible B lymphocyte. Cell **10**:27–34, 1977.
35. Wetzel GD, Kettman JR. Activation of murine B lymphocytes. III. Stimulation of B lymphocyte clonal growth with lipopolysaccharide and dextran sulfate. J Immunol **126**:723–728, 1981.
36. Kiyono H, McGhee JR, Wannemuehler MJ, Frangakis MV, Spalding DM, Michalek SM, Koopman WJ. *In vitro* immune response to a T cell dependent antigen by cultures of dissociated murine Peyer's patch. Proc Natl Acad Sci USA. **79**:596–600, 1982.
37. Vogel SN, Hilfiker ML, Caulfield MJ. Endotoxin-induced T lymphocyte proliferation. J Immunol **130**:1774–1779, 1983.
38. Hämmerling U. B-lymphocyte differentiation and regulatory signals. In: Siskind GW, Litwin SD, Weksler ME, eds. Developmental Immunobiology, New York, Grune and Stratton, pp59–83, 1979.
39. Abbott J, Ngiam K. Sequential expression of B lymphocyte surface antigens *in vitro*. Eur J Immunol **11**:411–417, 1981.
40. Boss M, Greaves M, Teich N. Abelson virus-transformed hemopoietic cell lines with pre-B cell characteristics. Nature (London) **278**:551–553, 1978.
41. Paige CJ, Kincade PW, Ralph P. A murine B cell leukemia line with inducible surface immunoglobulin expression. J Immunol **121**:641–647, 1979.
42. Boyd AW, Goding JW, Schrader JW. The regulation of growth and differentiation of a murine B cell lymphoma. J Immunol **126**:2461–2465, 1981.
43. Sultzer BM. Genetic analysis of lymphocyte activation by lipopolysaccharide endotoxin. Infect Immun **13**:1567–1584, 1976.
44. Watson J, Riblet R. Genetic control of responses to bacterial lipopolysaccharide in mice. II. A gene that influences a membrane component involved in the activation of bone marrow-derived lymphocytes by lipopolysaccharide. J Immunol **114**:1462–1468, 1975.
45. Nygren H, Dahlen G, Möller G. Bacterial lipopolysaccharides bind selectively to lymphocytes from lipopolysaccharide high-responder mouse strains. Scand J Immunol **10**:555–561, 1979.
46. Watson J, Kelly K, Whitlock C. Genetic control of endotoxin sensitivity. In: Schlesinger D, ed. Microbiology 1980. Washington, DC, Amer Soc Microbiol, pp4–10, 1980.
47. Jakobovits A, Sharon N, Zan-Bar I. Acquisition of mitogenic responsiveness by nonresponding lymphocytes upon insertion of appropriate membrane components. J Exp Med **156**:1274–1279, 1982.

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