

IMMUNOLOGIC PROPERTIES OF BACTERIAL LIPOPOLYSACCHARIDE (LPS)

II. The Unresponsiveness of C3H/HeJ Mouse Spleen Cells to LPS-Induced Mitogenesis is Dependent on the Method used to Extract LPS*

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There exists a unique strain of mouse, the C3H/HeJ, that is inherently refractive to many of the diverse biological effects of bacterial lipopolysaccharide (LPS)¹ which are characteristically displayed by LPS in other strains of mice. For example, the C3H/HeJ mouse is refractory to the capacity of LPS to non-specifically activate B lymphocytes (1, 2) as evidenced by the inability of spleen cells from this strain to support either LPS-induced mitogenic (3-6) or polyclonal antibody (6, 7) responses *in vitro*. This defect in the C3H/HeJ appears to be specific to LPS, since other B-cell mitogens, such as purified protein derivative of tuberculin (PPD), dextran sulfate, and poly I sponsor quantitatively normal mitogenic responses in this strain (3, 6, 7). The normally potent adjuvant effect of LPS on the immune response *in vivo* (8, 9) is also not seen in the C3H/HeJ mouse, as shown by the inability of LPS in this strain to enhance the antibody response to soluble bovine serum albumin (BSA) or to modulate the induction of a specific state of tolerance induced by deaggregated human IgG (HGG) into a specific state of immunity to HGG (5). The lack of these adjuvant effects in the C3H/HeJ mouse is not due to its inability to recognize the antigens, BSA and HGG, since the C3H/HeJ responds as well as other mouse strains when chal-

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¹ *Abbreviations used in this paper:* BH-LPS, base hydrolyzed LPS; BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; FCS, fetal calf serum; HGG, human IgG; KDO, 2-keto-3-deoxyoctulosonate; LPS, bacterial lipopolysaccharide; PPD, purified protein derivative of tuberculin.

lenged with immunogenic preparations of these antigens, namely, BSA emulsified in complete Freund's adjuvant or HGG aggregated by heat denaturation (5). In addition to these nonspecific effects of LPS on lymphocytes, LPS as a specific antigen is also highly immunogenic in most mouse strains (10, 11). However, the immune response to LPS in the C3H/HeJ differs strikingly from that observed in other strains, in that this response is relatively transient (5) and is elicited by only a comparatively narrow range of antigen doses (4). The LPS-specific defects in the C3H/HeJ also have been reported to include a high resistance to endotoxic shock as well as an abnormal pattern of leukocyte changes during an intraperitoneal inflammatory response to LPS (12-14).

These observations have led to the concept that the C3H/HeJ mouse is a nonresponder to a number of LPS-induced biological phenomena. However, this report will present more recent evidence, with regard to the mitogenic activity of LPS, which demonstrates that the unresponsive state inherent in the C3H/HeJ mouse to LPS-induced mitogenesis is a condition which depends on the method used to extract LPS. These results will be considered within the mechanistic framework that activation of B cells by LPS is critically dependent on the structural integrity of both the LPS molecule and putative B-cell receptors for LPS.

Materials and Methods

Mice. C57BL/6J and C3H/HeJ male mice, 6-8 wk of age, were obtained from Jackson Laboratories, Bar Harbor, Maine. C3H/St male mice, 6-8 wk of age, were obtained from the L. C. Strong Laboratory, Del Mar, Calif. Congenitally athymic (nude) mice were generously provided by Dr. Norman D. Reed at Montana State University, Bozeman, Mont., and were subsequently bred at Scripps Clinic and Research Foundation onto both C57BL/6J and C3H/HeJ backgrounds. At the time of these experiments, C57BL nudes were progeny of the second generation backcross onto the C57BL background. C3H/HeJ nudes (nu/nu) and heterozygotes (nu/+) after the third generation backcross were typed for their ability to mount in vitro mitogenic responses to LPS. This was accomplished using spleen cells obtained by partial splenectomy, kindly done by Dr. Sun Lee at Scripps. Only those mice proven to be nonresponders to LPS by this method were subsequently used for breeding. C3H/HeJ nudes were obtained by mating heterozygous females with nude males. All mice were maintained on Wayne Lab-Blox F6 pellets (Allied Mills, Inc., Chicago, Ill.) and chlorinated water acidified to a pH of 3.0 with HCl (15).

LPS. The preparations of LPS used are listed and described briefly in Table I. The details of their preparation are given elsewhere (16-20). Extraction of bacteria according to the method of Morrison and Leive (19) yielded preparations that were 97-100% LPS, 0-1% nucleic acid, and 0-2% protein. The purity of LPS obtained from other sources was not assessed. Some experiments utilized LPS isolated by re-extraction of *Escherichia coli* K235 LPS preparations that had been initially extracted by another procedure. Thus, *E. coli* K235 (phenol, butanol) was prepared by re-extraction of *E. coli* K235 (phenol-McIntire) LPS using the butanol-H₂O technique of Morrison and Leive (19). *E. coli* K235 (butanol, phenol) was prepared by re-extraction of *E. coli* K235 (butanol-Morrison) LPS using a procedure that duplicated as closely as possible the phenol-H₂O method described by McIntire et al. (20). In the latter case, LPS was suspended in H₂O at a concentration of 8.3 mg/ml and stirred with an equal volume of 60% phenol for 36 h at 4°C, after which the mixture was allowed to settle without stirring for 8 days at 4°C. The aqueous phase obtained after centrifugation at 3,000 *g* for 15 min was dialyzed overnight against H₂O, and then stirred with an equal volume of 88% phenol at 65°C for 15 min. After standing overnight at 4°C, the mixture was then centrifuged at 3,000 *g* for 15 min and the resultant aqueous phase was exhaustively dialyzed against H₂O and lyophilized.

Preparation of Lipid A. *E. coli* 055:B5 (TCA-Boivin) LPS was hydrolyzed for 90 min at 100°C in 1% glacial acetic acid (21). The precipitated lipid A was centrifuged at 900 *g* for 10 min, washed

once with hot 1% glacial acetic acid, washed again with hot distilled water, resuspended in H₂O at a concentration of approximately 1 mg/ml, and solubilized by addition of triethylamine (22) to a final concentration of 1%.

Preparation of Base Hydrolyzed LPS. Base hydrolyzed LPS (BH-LPS) was prepared from *E. coli* 055:B5 (TCA-Boivin) LPS by a modification of the method of Neter et al. (23) described previously (5).

Gel Filtration Chromatography. Sepharose 4B was packed and developed, under gravity flow, with a pressure head of less than one-half of the column height, with 0.12 M Tris buffer, pH 8.1. LPS was loaded onto columns at a concentration of 5–10 mg/ml in a volume no greater than 2% of the volume of the column bed. Fractions collected were dialyzed against normal saline and then assayed for LPS content and mitogenic activity. *E. coli* 0111:B4 LPS was quantitated in column fractions by colitose assay and *E. coli* K235 LPS by 2-keto-3-deoxyoctulosonate (KDO) assay.

Equilibrium Density Gradient Ultracentrifugation. Cesium chloride solutions having an average density of 1.40 g/cm³ were prepared in 0.12 M Tris buffer, pH 8.1, and contained samples of from 0.5 to 1.0 mg of LPS. Samples were centrifuged to equilibrium in the Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using a SW 50.1 rotor at 142,000 *g* for 60 h at 4°C, fractions collected, and densities determined from index of refraction measurements. Fractions were dialyzed against normal saline before assay of LPS content and mitogenic activity.

Deoxyhexose Assays. Colitose was quantitated using the procedure of Cynkin and Ashwell (24), after hydrolysis of samples in 0.2 N H₂SO₄ at 100°C for 15 min. KDO was quantitated as described by Waravdekar and Saslaw (25), after hydrolysis of samples in 0.04 N H₂SO₄ at 100°C for 8 min.

Culture Reagents. Supplemented minimal medium was prepared as follows: 100 ml contained 91.9 ml of Eagle's medium for suspension culture, 1.0 ml of 100 times Na pyruvate, 1.0 ml of 100 times nonessential amino acids, 0.1 ml of 100 times glutamine, 5.0 ml of fetal calf serum (lot no. 84167) (all of the above from Microbiological Associates, Albany, Calif.), and penicillin and streptomycin (final concentration of 100 U/ml and 100 µg/ml, respectively). Agents used in mitogenesis, including LPS, concanavalin A (Con A) (Miles-Yeda, Ltd., Rehovot, Israel), and PPD (Parke-Davis and Company, Detroit, Mich.) were sterilized by exposure of solutions to ultraviolet light.

Lymphocyte Suspensions. Spleen cell suspensions were prepared as described previously (5). These cells were either diluted with supplemented medium to a final density of 2×10^6 viable cells/ml and cultured directly, or first processed as follows to obtain purified T cells. The method used was based on that developed by Julius et al. (26). Briefly, a maximum of 5×10^8 spleen cells, suspended in 3 ml of balanced salt solution (BSS) containing 5% heat-inactivated fetal calf serum (FCS), were prefiltered at room temperature through sterilized glass wool columns to remove most dead cells and macrophages. These columns consisted of 12-ml plastic syringes packed to 5 ml with 400 mg of washed glass wool. The cells washed through the column with 20 ml of BSS plus FCS were centrifuged and resuspended at a concentration of 10^8 viable cells/ml, and a maximum of 2.5 ml was loaded onto sterilized nylon wool columns and washed with 1 ml of BSS plus FCS. These columns consisted of 12-ml plastic syringes packed to 9 ml with 800 mg of washed nylon wool (FT-242; Fenwall Laboratories, Morton Grove, Ill.). Columns were incubated at 37°C for 45 min, after which 13 ml of cells were collected from each column, washed, and resuspended in supplemented medium to a final density of 2×10^6 viable cells/ml.

Lymphocyte Cultures. Plastic tubes (no. 2054; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were seeded with 1 ml of cell suspension and placed, inclined at an angle of 5° from the horizontal, into a 37°C humidified incubator which maintained an atmosphere of 5% CO₂ in air. Cultures were fed daily with four drops of nutritional cocktail (27).

Measurement of DNA Synthesis. After the appropriate culture period, cells were incubated for 24 h with 1.0 µCi/ml of [³H]thymidine (0.1 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). The cells were then washed twice with cold phosphate-buffered normal saline, and precipitated with cold 5% TCA. TCA precipitates were collected and radioactive measurements made as described previously (5). Results are expressed as the arithmetic mean of duplicate or triplicate cultures.

Results

Mitogenic Activity of LPS from Different Sources. The relative mitogenic activity of LPS obtained from a wide variety of sources (see Table I) was assessed in cultures of spleen cells from C3H/St and C3H/HeJ mice. The different LPS preparations tested fell into two distinct categories: those to which the C3H/HeJ was a nonresponder, and those to which this strain was a low to a high responder. LPS preparations to which the C3H/HeJ was a nonresponder are shown in Fig. 1. In this experiment, spleen cells were incubated in the presence of LPS obtained from three different sources, and the stimulation of DNA synthesis was measured at 2 days of culture after a 24 h incubation with [³H]TdR. The results demonstrate that, as we described previously (5), the C3H/HeJ is totally unresponsive to LPS from *E. coli* K235 at concentrations ranging from 0.1 μ g to 100 μ g/ml. The C3H/HeJ is also unresponsive to LPS from two other sources, namely, the rough mutants, *Salmonella minnesota* Re 595 and *E. coli* 0111:B4-J5. On the other hand, all three preparations were highly stimulatory for spleen cells obtained from C3H/St mice.

In contrast to these LPS preparations which were "negative" in inducing mitogenic responses in the C3H/HeJ, other preparations were found which were "positive" in their capacity to induce mitogenic responses in this strain. These are listed in Table II. The stimulation shown was obtained under conditions of LPS concentration and day of harvest determined to be optimal for each strain of mouse and source of LPS. The negative LPS preparations described in Fig. 1 are shown again for comparison with the positive preparations. These positive preparations include LPS obtained from *E. coli* 0111:B4 by four different extraction methods, and, in addition, LPS obtained from *E. coli* 055:B5 and *E. coli* 0113. Each of these preparations sponsored significant stimulation of C3H/HeJ cells. However, it is apparent that the magnitude of this stimulation was always lower than that observed with C3H/St cells, covering a broad range of values from as low as 5% of the C3H/St response, obtained with a phenol extract of *E. coli* 0111:B4, to as high as 75% of the C3H/St response, seen with a butanol extract of *E. coli* 0111:B4. This latter finding demonstrates that the ability of cells from the C3H/HeJ to respond mitogenically to different preparations of positive LPS is more dependent on the technique used to extract LPS, than on the strain of bacteria from which it is obtained.

These initial observations prompted the following series of experiments designed to establish, (a) whether the unique capacity of positive LPS preparations to activate C3H/HeJ cells is actually due to LPS or to a non-LPS contaminant; (b) how stimulation by positive LPS in the C3H/HeJ compares qualitatively and quantitatively to that seen with this LPS in other mouse strains; and, (c) if the LPS extraction method is also important in determining whether LPS is totally negative in its mitogenic activity in the C3H/HeJ mouse.

Stimulation by a Positive Preparation is Attributable to LPS. In order to determine whether the mitogenic material in a positive preparation is LPS or a contaminant, the following two experiments were carried out. First, a purified butanol preparation from *E. coli* 0111:B4 was subjected to gel filtration on Sepharose 4B, and fractions were individually assayed for mitogenic activity for both C3H/St and C3H/HeJ spleen cells, and quantitated for their content of LPS.

TABLE I
Extraction and Source of LPS Preparations

Bacterial strain	Method of extraction	Source	Abbreviation
<i>S. minnesota</i> Re 595	Phenol-chloroform-petroleum ether (PCP) (16)	Scripps	PCP-Galanos
<i>E. coli</i> 055:B5	TCA (17)	Difco	TCA-Boivin
<i>E. coli</i> 0113	Phenol-water (18)	Dr. J. Rudbach	Phenol-Westphal
<i>E. coli</i> 0111:B4	Phenol-chloroform-petroleum ether (16)	Scripps	PCP-Galanos
<i>E. coli</i> 0111:B4	Butanol-water (19)	Scripps	Butanol-Morrison
<i>E. coli</i> 0111:B4	Phenol-water (19)	Scripps	Phenol-Morrison
<i>E. coli</i> 0111:B4	Phenol-water (18)	Difco	Phenol-Westphal
<i>E. coli</i> 0111:B4	TCA (17)	Difco	TCA-Boivin
<i>E. coli</i> K235	Phenol-water (20)	Dr. F. McIntire	Phenol-McIntire
<i>E. coli</i> K235	Butanol-water (19)	Scripps	Butanol-Morrison
<i>E. coli</i> K235	Phenol-water (20), then Butanol-water (19)*	Dr. F. McIntire, and Scripps	Phenol, Butanol
<i>E. coli</i> K235	Butanol-water (19), then Phenol-water (20)*	Scripps	Butanol, Phenol

* These preparations were obtained by two sequential extractions: bacteria were initially extracted by the first method listed, followed by re-extraction of the LPS using the second method listed. See Materials and Methods.

The latter was determined by assaying for the presence of colitose, since this deoxyhexose is the principal sugar of the O-antigenic polysaccharide of this bacterial strain (28), and, moreover, is known to exist in nature principally as a constituent of gram-negative bacterial lipopolysaccharides (29). Thus, this assay is highly specific for LPS. The results of this experiment, shown in Fig. 2, demonstrate that the chromatographic profile obtained for mitogenic activity was essentially superimposable on the profile obtained for LPS content. These data strongly suggest that the mitogenic material is LPS.

The mitogenic material was also correlated with an additional physical property of LPS, namely its partial specific volume. A sample of the peak Sepharose 4B fraction from Fig. 2 was subjected to CsCl equilibrium density gradient ultracentrifugation, and, as seen in Fig. 3, the profile obtained for mitogenic activity in both mouse strains was again superimposable on that obtained for LPS content. Moreover, the density of 1.42 g/cm³ estimated from the peak CsCl fraction is within the range that is characteristic of LPS.

Therefore, by two independent and sequential purification procedures, the mitogenic activity and LPS coisolated, demonstrating that LPS is in fact the biologically active substance in a positive preparation.

Characterization of the Parameters of the Mitogenic Response to Positive LPS. The establishment that mitogenic stimulation of C3H/HeJ spleen cells by a positive preparation is attributable to LPS, permitted the further characterization of the parameters of this response.

DOSE-RESPONSE PROFILE. Spleen cells from the C3H/HeJ are less responsive to positive LPS than those from the C3H/St, at all LPS concentrations tested. This can be seen in Fig. 4 which depicts the dose-response profile of the

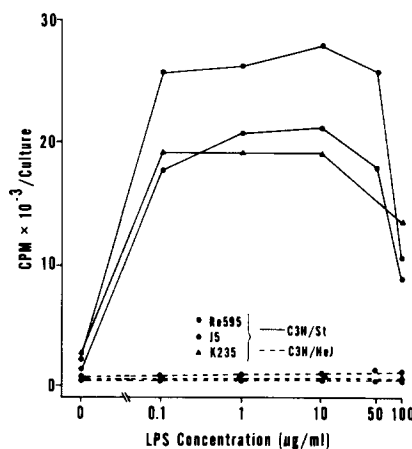


FIG. 1. Relative ability of C3H/St and C3H/HeJ spleen cells to respond mitogenically to LPS. The peak mitogenic response (day 2) is shown for spleen cells from C3H/St (—) and C3H/HeJ (---) mice to the following LPS preparations: *S. minnesota* Re 595 (PCP-Galanos) (●), *E. coli* 0111:B4-J5 (PCP-Galanos) (○), and *E. coli* K235 (phenol-McIntire) (▲).

mitogenic stimulation, assayed on day 2 of culture, induced by *E. coli* 0111:B4 (butanol-Morrison) LPS. It is evident that at each concentration of LPS tested the level of stimulation of cells from the C3H/St was greater than that observed with cells from the C3H/HeJ.

KINETIC PROFILE. The mitogenic response to positive LPS in the C3H/HeJ is relatively transient in comparison to that observed in the C3H/St. The kinetic profile of the mitogenic response to optimal concentrations of *E. coli* 0111:B4 (butanol-Morrison) LPS shows that C3H/St spleen cells responded well on both days 2 and 3 of culture, whereas C3H/HeJ spleen cells responded well on day 2, but very poorly on day 3 (Fig. 5). A kinetic difference between these two mouse strains has been consistently observed with this as well as with other positive LPS preparations. However, although the peak response by C3H/HeJ cells always occurs on day 2 and declines sharply on day 3, the peak response by C3H/St cells in some experiments also occurs on day 2, but, in contrast to the C3H/HeJ, declines very little on day 3 (data not shown).

STRUCTURAL STUDIES. Structural studies of LPS revealed that the mitogenic activity of positive LPS for C3H/HeJ mice is attributable to the lipid A region of the molecule. This was shown by the fact that base hydrolysis of *E. coli* 055:B5 (TCA-Boivin) LPS, a treatment that removes the ester-linked fatty acids from lipid A without affecting the chemical structure of the O-antigenic polysaccharide (5, 30), resulted in a 95% loss in mitogenic activity of this positive LPS for both C3H/St and C3H/HeJ cells (Table III). Furthermore, isolated lipid A itself, obtained by mild acid hydrolysis of positive LPS, was mitogenic for both mouse strains.

CELLULAR SPECIFICITY OF THE MITOGENIC RESPONSE. The mitogenic stimulation of C3H/HeJ spleen cells by selected preparations of LPS is apparently restricted to activation of B lymphocytes. This conclusion is based on the following two observations. First, spleen cells obtained from nude mice, bred onto a C3H/HeJ background, were mitogenically stimulated by positive LPS,

TABLE II
 Classification of LPS Preparations as "Positive" or "Negative" for Mitogenic Stimulation
 of C3H/HeJ Spleen Cells*

LPS source	C3H/St		C3H/HeJ	
	-LPS	+LPS	-LPS	+LPS
	<i>cpm/culture</i>			
Negative LPS				
<i>E. coli</i> K235 (phenol-McIntire)	3,140	35,180	880	920
<i>E. coli</i> 0111:B4-J5 (PCP-Galanos)	1,450	21,310	620	690
<i>S. minnesota</i> Re 595 (PCP-Galanos)	2,390	27,870	940	1,640
Positive LPS				
<i>E. coli</i> 0111:B4 (butanol-Morrison)	1,450	34,350	620	24,270
<i>E. coli</i> 0111:B4 (TCA-Boivin)	2,010	37,160	620	12,990
<i>E. coli</i> 0111:B4 (phenol-Westphal)	1,480	32,000	350	4,350
<i>E. coli</i> 0111:B4 (phenol-Morrison)	1,100	25,460	170	1,430
<i>E. coli</i> 055:B5 (TCA-Boivin)	2,010	31,170	620	8,070
<i>E. coli</i> 0113 (phenol-Westphal)	2,390	34,400	940	4,730

* These data represent the maximal responses determined individually for each strain of mouse and preparation of LPS, and are expressed numerically rather than as a series of kinetic and dose-response profiles.

but not by negative LPS (Fig. 6). Both LPS preparations, however, were mitogenic for spleen cells obtained from nude mice bred onto other backgrounds, typified in this experiment by the C57BL strain. It can also be seen that C3H/HeJ nude spleen cells are capable of responding to another B-cell mitogen, namely PPD.

In contrast to the stimulation observed with positive LPS using nude spleen cells, the mitogenic effect of both positive and negative LPS was negligible when tested on peripheral T cells purified from either C3H/St or C3H/HeJ spleens by the nylon wool technique (Fig. 7). These T cells, however, were readily activated by the T-cell mitogen, Con A. Taken together, the results presented in Figs. 6 and 7 demonstrate that LPS is a specific B-cell mitogen in the C3H/HeJ.

In summary, the stimulation of C3H/HeJ spleen cells by a positive LPS preparation appears to be qualitatively similar to that obtained in other mouse strains, at least as assessed by the above criteria, although quantitatively this response is lower and more transient.

Importance of the Method of Extraction of LPS from Bacteria. Since the experiments described thus far clearly demonstrate that the C3H/HeJ mouse is competent to respond mitogenically to some preparations of LPS, an important question is now, why are similar responses not elicited by other LPS preparations which do, nonetheless, have the capacity to stimulate lymphoid cells from other mouse strains? A possible answer is suggested by the results in Table II, which demonstrate that positive LPS obtained by butanol extraction of *E. coli* 0111:B4 is nearly 20 times more mitogenic in the C3H/HeJ than positive LPS obtained by phenol extraction from this same bacterial strain. Since the relative mitogenic activity of these positive LPS preparations in the C3H/HeJ appears to

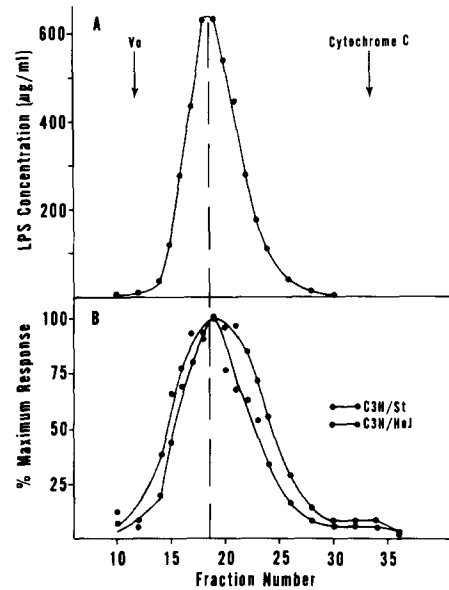


FIG. 2. Gel filtration of *E. coli* 0111:B4 (butanol-Morrison) LPS on Sepharose 4B. The concentration of LPS in individual fractions was determined by colitose content (A). The peak fraction was diluted by the appropriate factor to give a concentration of 200 µg/ml of LPS. Other fractions were diluted by this same factor and 50 µl of each was added to individual 1 ml cultures to assess their mitogenic activity (B). Results are means of triplicate cultures and are expressed as the percent of the response obtained with the peak column fraction in each mouse strain. C3H/St (●), C3H/HeJ (○).

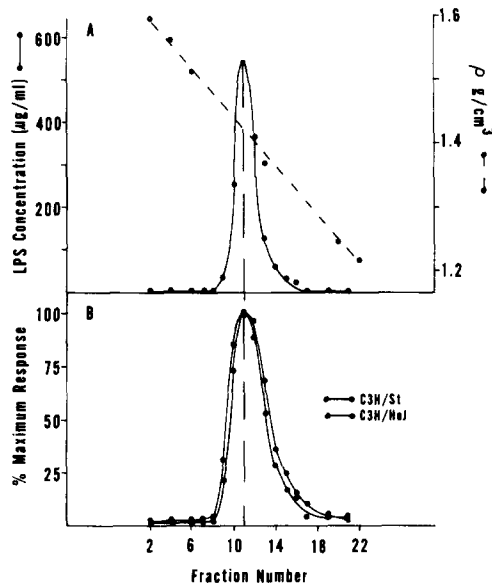


FIG. 3. Equilibrium density gradient ultracentrifugation of *E. coli* 0111:B4 (butanol-Morrison) LPS on CsCl. Details are identical to those described in legend to Fig. 2, except that the peak gradient fraction was diluted to 100 µg/ml. (A) Assays of LPS concentration (●—●) and CsCl densities (○---○). (B) Assays of mitogenic activity in C3H/St (●) and C3H/HeJ (○).

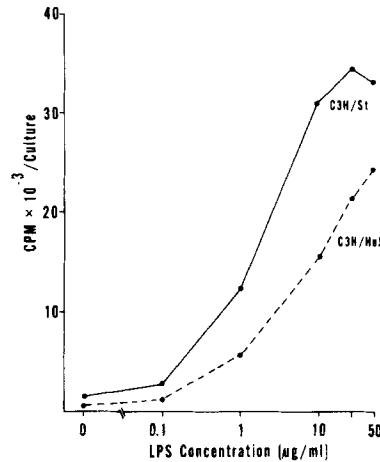


FIG. 4. Relative ability of C3H/St and C3H/HeJ spleen cells to respond mitogenically to a positive LPS; dose-response profile. The peak mitogenic response (day 2) to *E. coli* 0111:B4 (butanol-Morrison) LPS is shown. (●—●), C3H/St; (○---○), C3H/HeJ.

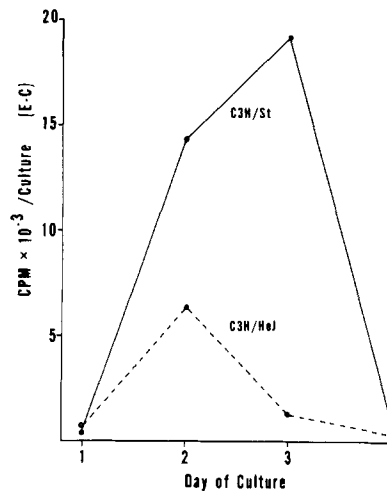


FIG. 5. Relative ability of C3H/St and C3H/HeJ spleen cells to respond mitogenically to a positive LPS; kinetic profile. The mitogenic response to 10 µg/ml of *E. coli* 0111:B4 (butanol-Morrison) LPS is shown. (●—●), C3H/St; (○---○), C3H/HeJ.

be determined by the extraction technique, it is likely that the extraction technique is also what determines whether LPS will be totally negative in its mitogenic capacity in the C3H/HeJ.

In order to test this concept, two preparations of LPS, each isolated from *E. coli* K235, were initially tested for mitogenic stimulation of C3H/St and C3H/HeJ spleen cells: LPS extracted by McIntire with phenol (*E. coli* K235 [phenol-McIntire] LPS), and LPS extracted in our laboratory with butanol (*E. coli* K235 [butanol-Morrison] LPS). The results depicted in Fig. 8 demonstrate that, although the phenol-prepared LPS was negative for C3H/HeJ spleen cells, as shown earlier (Fig. 1), butanol-prepared LPS was clearly positive, giving a response in the C3H/HeJ that was 80% of that observed in the C3H/St. In the

TABLE III
Mitogenic Activity of Positive LPS is Attributable to the Lipid A Region

Treatment*	C3H/St	C3H/HeJ
	<i>cpm/culture</i> ‡	
Saline	3,240	620
LPS	29,420	9,310
BH-LPS	4,470	950
Lipid A	36,160	13,370

* Spleen cells were cultured for 2 days in the presence of either saline or 10 $\mu\text{g/ml}$ of LPS, BH-LPS, or lipid A; all from *E. coli* 055:B5 (TCA-Boivin).

‡ Results are expressed as the mean of duplicate cultures.

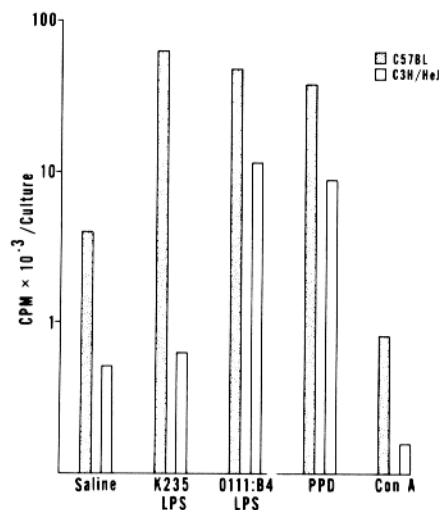


FIG. 6. Cellular specificity of mitogenesis induced by a positive LPS; nude spleen cells. Spleen cells obtained from nude C57BL or C3H/HeJ mice were incubated for 2 days (peak response) in the presence of saline, 25 $\mu\text{g/ml}$ of *E. coli* K235 (phenol-McIntire) LPS, 20 $\mu\text{g/ml}$ of *E. coli* 0111:B4 (butanol-Morrison) LPS, 200 $\mu\text{g/ml}$ of PPD, or 2 $\mu\text{g/ml}$ of Con A.

C3H/St, this butanol extract was more mitogenic than the phenol extract when optimum concentrations of each were compared. These optimum concentrations were also found to be different, in that the phenol extract induced maximum stimulation from between 1 and 10 $\mu\text{g/ml}$ of LPS, whereas with the butanol extract maximum stimulation occurred at 100 $\mu\text{g/ml}$ of LPS. In the C3H/HeJ, this high concentration of butanol-prepared LPS was also the optimum dose. It is unlikely that the stimulation induced by butanol-prepared *E. coli* K235 LPS is due to the extraction of a mitogenic contaminant, since, as previously demonstrated for *E. coli* 0111:B4 (butanol-Morrison) LPS (Fig. 3), the mitogenic activity of this butanol extract coequilibrium banded with LPS on CsCl gradients. (B. J. Skidmore, unpublished observation).

Two possible explanations might account for the striking difference between these two LPS preparations. First, this difference could be due to the presence of

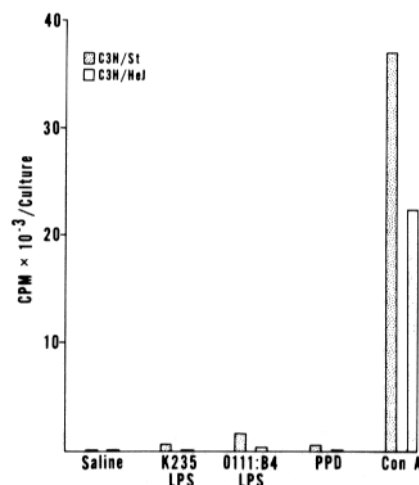


FIG. 7. Cellular specificity of mitogenesis induced by a positive LPS; peripheral T cells. The day 2 (peak) mitogenic response is shown for purified peripheral T cells obtained from spleens by the nylon wool technique. Mitogens are described in legend to Fig. 6.

a C3H/HeJ-specific inhibitor of LPS-induced mitogenesis in preparations of *E. coli* K235 (phenol-McIntire) LPS, which may be absent from preparations of *E. coli* K235 (butanol-Morrison) LPS. Secondly, it is possible, instead, that the phenol treatment may cause chemical and/or conformational alterations in the LPS which result in a total loss of its mitogenic activity in the C3H/HeJ, but in only a partial loss in the C3H/St. In order to distinguish between these two possibilities, the effect of re-extraction of LPS on its mitogenic activity was assessed. That is, *E. coli* K235 (phenol-McIntire) LPS was re-extracted with butanol to remove the putative inhibitor from the LPS preparation, or, *E. coli* K235 (butanol-Morrison) LPS was re-extracted with phenol to induce the presumptive alterations in the LPS molecule.

The results of such experiments, depicted in Fig. 8, clearly show that *E. coli* K235 (phenol-McIntire) LPS after re-extraction with butanol (*E. coli* K235 [phenol, butanol] LPS) was still negative in the C3H/HeJ, although it retained its mitogenic activity in the C3H/St. In contrast, *E. coli* K235 (butanol-Morrison) LPS after re-extraction with phenol (*E. coli* K235 [butanol, phenol] LPS), according to the method of McIntire et al. (20), totally lost its capacity to mitogenically stimulate C3H/HeJ cells. This LPS, however, was still capable of stimulating cells from the C3H/St, although less stimulation was obtained than with the starting material, that is *E. coli* K235 (butanol-Morrison) LPS. In fact, the magnitude of the response was reduced essentially to the level observed with the other two preparations of *E. coli* K235 LPS isolated by procedures using phenol. In other words, by re-extraction with phenol it is possible to directly convert a positive LPS into a negative LPS.

In summary, these data firmly establish that it is the method by which LPS is extracted and not the strain of bacteria from which it is isolated, that determines whether LPS will be positive or negative in its mitogenic capacity.

Inhibitory Activity of Negative LPS. Although negative LPS has lost its ability to stimulate C3H/HeJ B cells, it appears to have retained its ability to

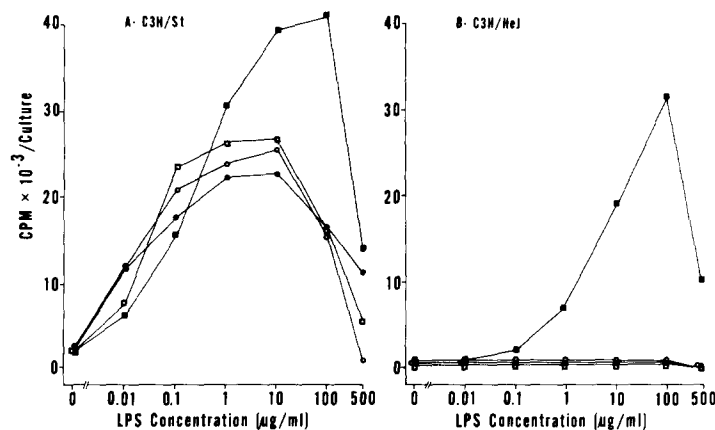


FIG. 8. Relative ability of different preparations of *E. coli* K235 LPS to mitogenically stimulate C3H/St and C3H/HeJ spleen cells. LPS obtained from three different sources, *E. coli* K235 (phenol-McIntire) (●), *E. coli* K235 (butanol-Morrison) (■), *E. coli* K235 (phenol, butanol) (○), and *E. coli* K235 (butanol, phenol) (□), were assessed for mitogenic activity in cultures of C3H/St (A) and C3H/HeJ (B) spleen cells. The peak (day 2) response is shown for both strains.

block stimulation of these B cells by positive LPS. This conclusion is based on the following experiment. Spleen cells obtained from C3H/HeJ mice were incubated with a negative LPS, *E. coli* K235 (phenol-Morrison), for 2 h at 37°C, and subsequently (without washing) with a positive LPS, *E. coli* 0111:B4 (TCA-Boivin), for 2 days at 37°C, at which time the mitogenic response was assessed. It can be seen (Fig. 9) that negative LPS caused a dose-dependent inhibition of stimulation induced by a positive LPS that was not due to nonspecific toxicity, since there was no detectable inhibition of a PPD mitogenic response.

Discussion

The unresponsive state inherent in the C3H/HeJ mouse strain to LPS-induced mitogenesis is a condition which the present studies show to be restricted to only certain preparations of LPS. LPS prepared by the phenol-H₂O method of McIntire et al. (20) or the phenol-chloroform-petroleum ether method of Galanos et al. (16) were negative in their capacity to mitogenically stimulate C3H/HeJ spleen cells, although these same preparations were highly stimulatory for cells from the C3H/St mouse. In contrast, other preparations exist which are positive for mitogenic activity in the C3H/HeJ strain. These include LPS extracted by the phenol-H₂O methods of Westphal and Jann (18) or Morrison and Leive (19), although these phenol preparations sponsored only weak stimulation of the C3H/HeJ. The most active preparations in this strain were those obtained by the TCA method of Boivin et al. (17), and in particular, those obtained by the butanol method of Morrison and Leive (19). LPS extracted by this butanol method consistently induced mitogenic responses in spleen cells from the C3H/HeJ that were as high as 70–80% of those observed in cells from the C3H/St. The stimulation observed with positive preparations is most likely not due to the presence of a non-LPS contaminant, since the mitogenic activity of

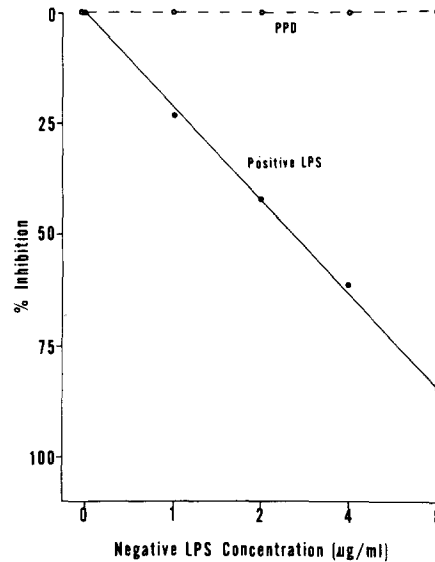


FIG. 9. Inhibitory effect of preincubation of C3H/HeJ spleen cells with a negative LPS on the mitogenic response to a positive LPS. Spleen cells were incubated with *E. coli* K235 (phenol-McIntire) LPS for 2 h at 37°C, and then, without washing, for 2 days at 37°C with either 4 µg/ml of *E. coli* 0111:B4 (TCA-Boivin) LPS or 200 µg/ml of PPD.

the butanol extract of *E. coli* 0111:B4 was demonstrated to cochromatograph with LPS on column chromatography and subsequently to coequilibrium band with LPS on CsCl. It is difficult to rule out completely, however, the possibility that a highly active contaminant may be present, tightly bound to LPS. Moreover, it is not known whether the stimulation observed with crude commercial extracts is also due exclusively to LPS.

The mitogenic responses to positive LPS in the C3H/St and C3H/HeJ appear to be qualitatively similar to each other as judged by the following two criteria. First, in the C3H/HeJ strain, the mitogenic activity of LPS was a property shown to be attributable to the lipid A region of the molecule, which is the same region in which the mitogenic activity of LPS has been reported to reside for the stimulation of lymphoid cells from other mouse strains (31–34). Secondly, in the C3H/HeJ, the mitogenic activity of LPS was shown to be specific for B lymphocytes, as it is in other strains of mice (1, 2). In contrast to these similarities, the mitogenic responses to positive LPS in the C3H/St and C3H/HeJ differ quantitatively from each other. At all LPS concentrations tested, the magnitude of the stimulation induced by positive LPS in the C3H/HeJ was less than that obtained in the C3H/St. A quantitative difference was also seen when the kinetic profiles of the mitogenic responses were compared. That is, the response in the C3H/HeJ was observed to decline rapidly from day 2 to 3 of culture, whereas the response in the C3H/St either reached a peak on day 3, or peaked on day 2 and declined very little on day 3.

The technique used to extract LPS was shown to be the critical factor in determining whether LPS preparations are positive or negative for mitogenic activity in the C3H/HeJ mouse. This was demonstrated most clearly by experi-

ments in which LPS was prepared by two different procedures, but using as starting material the identical bacterial strain. *E. coli* K235 (phenol-McIntire) LPS was found to be completely nonstimulatory in the C3H/HeJ, whereas, *E. coli* K235 (butanol-Morrison) LPS was highly stimulatory. The fact that re-extraction of the inactive phenol-prepared LPS with butanol did not restore its activity would seem to rule out the possibility of inhibitors. The reverse procedure, however, that is re-extraction of the active, butanol-prepared LPS with phenol, selectively destroyed its mitogenic activity in the C3H/HeJ, while merely lowering its activity in the C3H/St to the level displayed by other phenol extracts of *E. coli* K235 in this mouse strain. In view of these data, it is significant that other published reports describing the C3H/HeJ mouse as a nonresponder to LPS-induced mitogenesis (3-7) were also obtained using phenol-prepared LPS. Not all LPS preparations isolated by procedures utilizing phenol were negative in the present studies. The inhibitory effect of phenol on LPS may vary greatly depending on the concentration, temperature, and duration of the phenol treatment, conditions which differ among extraction techniques. The negativity of *E. coli* K235 (phenol-McIntire) LPS in the C3H/HeJ mouse is most likely related to the fact that this LPS was obtained by a procedure (20) in which extraction with phenol was carried out for a prolonged period of time, and at a high temperature.

The obvious question raised by these experiments is why stimulation of C3H/HeJ B cells by LPS is dependent on the method by which LPS is extracted. In view of the possible modulatory effect of T cells and macrophages on LPS-induced mitogenesis (35, 36), these accessory cell types may, in some way, dictate to B lymphocytes the panel of LPS preparations to which they can respond. However, *in vitro* experiments have revealed neither an enhancing nor an inhibitory effect of admixing spleen cells obtained from responder strain mice and C3H/HeJ mice on the mitogenic response to either a negative (reference 7 and footnote 2) or to a positive LPS.² Therefore, the defect in the C3H/HeJ that limits mitogenic responses to certain LPS preparations and which renders it totally unresponsive to other LPS preparations appears to be due to an intrinsic defect in B lymphocytes.

More precise information about the nature of this B-cell defect is provided by the present studies. Activation of B lymphocytes by LPS most likely involves a complex sequence of events. It is safe to assume that the minimal requirement for this activation, as well as the initial step, is the binding of LPS to the B-cell surface, perhaps to a specific LPS receptor analogous to that found on human erythrocytes (37). The question then becomes whether C3H/HeJ B cells are refractory to mitogenesis induced by negative LPS simply because these cells are devoid of receptors for negative LPS. Two observations suggest that this is not the case. First, the finding that negative LPS blocks stimulation of C3H/HeJ B cells induced by positive LPS, could be interpreted as evidence that these receptors have retained their capacity to bind negative LPS, although it should be stressed that the precise mechanism by which this inhibition occurs has not

² Skidmore, B. J., J. M. Chiller, and W. O. Weigle. 1975. Immunologic properties of LPS. IV. Cellular basis of the unresponsiveness of C3H/HeJ mouse spleen cells to LPS-induced mitogenesis. Manuscript in preparation.

been delineated. Secondly, recent experiments by Watson and Riblet (7) have shown equivalent uptake of a radioactively labeled preparation of nonstimulatory LPS by responder and nonresponder spleen cells. Taken together, these observations suggest that the receptors for negative LPS may not be reduced in number on C3H/HeJ B cells, but that the structure of these receptors may be altered, so that the binding of negative LPS to these receptors is essentially a sterile interaction in that it does not lead to cell activation. The altered LPS receptors may be the phenotypic expression of the genetic defect in the C3H/HeJ mouse that is believed to be responsible for its refractory state to LPS-induced mitogenesis (4).

The unique capacity of positive LPS to mitogenically activate cells from the C3H/HeJ could be explained if the binding of positive LPS to these altered receptors, in contrast to the binding of negative LPS, was able to initiate stimulation. Alternatively, an additional class of LPS receptors may exist, unaltered in the C3H/HeJ, through which activation by positive, but not by negative LPS is mediated. Both of these possibilities imply that structural differences exist between positive and negative LPS preparations (*vide infra*). These differences would be present at sites on the LPS molecule that are important in order for the binding of LPS to receptors to initiate mitogenesis. In contrast, these sites would have no quantitative influence on the binding of LPS *per se* to altered or unaltered receptors for either positive or negative LPS. These sites on the LPS molecule are presumed to be labile, and as a result, are altered from their native structure in certain extraction reagents, especially phenol, whereas they remain essentially unaltered in milder extraction reagents, particularly butanol. Accordingly, negative LPS should be regarded as a structurally altered molecule, while positive LPS should be viewed as a relatively unaltered molecule.

Altered LPS molecules and altered LPS receptors form the basis of the following models which attempt to account for the differences between the mitogenic activities of positive and negative LPS in C3H/St and C3H/HeJ mice (Fig. 10).

1 LPS-Responsive B-Cell Population:1 Class of LPS Receptors. Even a normal LPS receptor, such as that found on cells from the C3H/St, may have the ability to discriminate between positive and negative LPS molecules on the basis of their presumptive structural differences. Because of the alterations present in negative LPS, the binding of this preparation to the receptor would give a lower mitogenic response than the binding of positive LPS. An altered LPS receptor, such as found on B cells from the C3H/HeJ, may be even more discriminatory such that the binding of negative LPS to the receptor totally fails to initiate stimulation, and the binding of positive LPS initiates a response that is less than that seen in the C3H/St. In other words, interaction between, (a) an unaltered LPS and a unaltered receptor results in optimum stimulation; (b) either an altered LPS or an altered receptor gives intermediate stimulation; and (c) an altered LPS and an altered receptor initiates no stimulation.

1 LPS-Responsive B-Cell Population:2 Classes of LPS Receptors. Two classes of LPS receptors may be present on B cells. In the C3H/St, the binding of positive LPS to these receptors leads to stimulation initiated through both

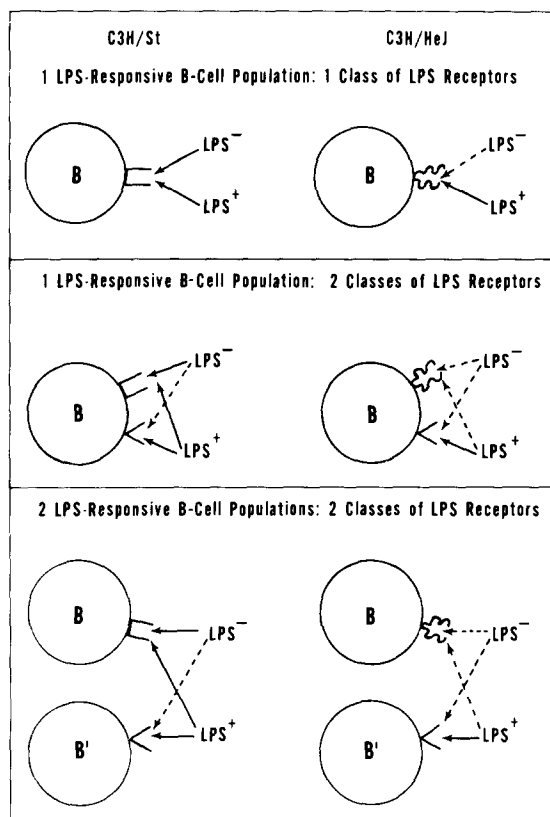


FIG. 10. Models accounting for relative mitogenic activities of positive and negative LPS for spleen cells from C3H/St and C3H/HeJ mice. LPS⁻ and LPS⁺ are negative and positive preparations, respectively. Solid arrows indicate LPS binding that leads to mitogenesis, dashed arrows indicate LPS binding that fails to lead to mitogenesis. Unaltered (native) LPS receptors (\square and $<$), altered LPS receptor (\odot).

classes, thereby giving an optimum response. However, binding of altered, negative LPS to both receptors may initiate stimulation through only one class, thus producing an intermediate response. In the C3H/HeJ, only this latter receptor class would be altered, thereby abolishing the response to negative LPS. The response to positive LPS would be intermediate, since in the C3H/HeJ stimulation by this LPS may be mediated by only the unaltered receptor class.

2 LPS-Responsive B-Cell Populations: 2 Classes of LPS Receptors. Recent evidence suggests that the B-lymphocyte population may be composed of subsets of B cells, each displaying a unique specificity for different mitogens (38-40). Therefore, the two hypothetical classes of LPS receptors proposed above may be expressed on two separate B-cell subpopulations.

These models will account equally well for the data given in the present studies, but the information does not exist that allows one to distinguish among them. However, each makes simple and readily testable predictions which should permit the eventual elucidation of the mechanism.

Since each of these models relies on the assumption that defined structural

differences between positive and negative LPS account, in part, for their dissimilar mitogenic activities, it is important at this juncture to ask whether such structural differences do in fact exist. Preliminary evidence indicates that, as compared to *E. coli* K235 (butanol-Morrison) LPS, *E. coli* K235 (phenol-McIntire) LPS shows a significant shift to a higher equilibrium density in CsCl gradients. It is of interest that a similar difference was previously reported to exist between LPS extracted from *E. coli* 0111:B4 by phenol or butanol (19); preparations shown in the present studies to differ vastly in their mitogenic activity in the C3H/HeJ. The effect of phenol on density was irreversible, since no change in this property was observed when phenol-prepared *E. coli* 0111:B4 LPS was re-extracted with butanol (19). The fact that the effect of phenol on the mitogenic property of LPS was also irreversible (Fig. 8), emphasizes the possibility that these two properties may be closely related. Denaturation of DNA (41) and of BSA (42) is known to increase their buoyant density, suggesting that phenol extraction may "denature" LPS, while butanol extraction may do so to a lesser extent. However, since buoyant density is affected by a number of factors, such as net solvation and true anhydrous density (43), as well as interaction with other molecules (44), the exact significance of this density change must await a more precise chemical and physical characterization of positive and negative LPS preparations. The lipid A moiety of the LPS molecule is the region in which differences between these preparations are presently being sought, since, as described earlier, the mitogenic activity of positive LPS was shown to be a function of lipid A in both the C3H/St and C3H/HeJ strains. Furthermore, the inability of negative LPS preparations to induce mitogenesis in the C3H/HeJ is most likely also directly attributable to lipid A rather than to a presumptive inhibitory effect of the polysaccharide region. This conclusion is based on the fact that two of the negative preparations used were extracted from mutant bacteria which synthesize LPS molecules deficient in polysaccharide; namely, *E. coli* 0111:B4-J5 LPS which lacks most of the core and all of the O-antigenic regions (45), and *S. minnesota* Re 595 LPS which is devoid of both the core and antigenic polysaccharides, and consists of only lipid A and KDO (46). It is conceivable, for example, that lipid A's of positive and negative LPS preparations possess distinct differences in fatty acid and/or PO₄⁻ content, which may be directly responsible for the differences in their mitogenic activity.

The effect that the method of extraction of LPS from whole bacteria, or from bacterial cell walls, can have upon the subsequent composition and biological activity of LPS, has in fact been described by others and cannot be overstressed. A number of chemical procedures exist for the isolation and purification of bacterial lipopolysaccharides. Each of these procedures has both advantages and disadvantages with respect to relative purity from contaminating bacterial products and risk of chemical degradation of the LPS due to the reagents involved in the isolation procedures. Recently, Wober and Alaupović (47) have suggested that two of the methods frequently employed in the extraction of LPS, that is, phenol and TCA, may cause a selective cleavage of LPS at high temperatures. They further proposed that phenol "unfolds the LPS structure" and thus causes maximal exposure to susceptible linkages such as ester- and amide-bound fatty acids. Further studies have demonstrated chemical degrada-

tion of the LPS from *S. marcescens* by treatment with aqueous phenol, both of the O-specific side chains and of the ester-linked fatty acids (48). These authors conclude that an intact lipid moiety of the LPS molecule cannot be isolated from LPS preparations obtained by phenol extraction procedures. Recent studies by Chang and Nowotny (49) have characterized a number of lipid by-products resulting from acid hydrolysis of LPS preparations and have emphasized the importance of the chemical procedure used to isolate lipid components on the resultant chemical composition of the lipid ultimately obtained. Structural and/or chemical alterations in preparations of LPS resulting from the method of extraction and subsequent purification may affect other biological properties of LPS besides its mitogenic activity. Nowotny et al. (50) very early observed that treatment of crude TCA extracts of LPS with hot-aqueous phenol, while resulting in a substantial purification of the LPS, significantly decreased the toxicity of such preparations.

In this regard, it will be of interest to determine whether the refractory state of the C3H/HeJ mouse strain to activities of LPS other than mitogenesis is also restricted to only selected LPS preparations. For example, in the C3H/HeJ, does *E. coli* K235 (butanol-Morrison) LPS, in contrast to *E. coli* K235 (phenol-McIntire) LPS, have the capacity to function as a toxin, an adjuvant of antibody formation, a potent immunogen, or as an activator of polyclonal antibody synthesis? Such an analysis, in concert with the elucidation of the presumptive physical and chemical properties of positive and negative LPS, should provide a fresh experimental approach to the characterization of the structure-function relationships involved in the broad spectrum of biological activities of LPS.

Summary

The C3H/HeJ mouse strain, previously shown to be a nonresponder to bacterial lipopolysaccharide (LPS)-induced mitogenesis *in vitro*, was demonstrated by the present studies to be competent to respond mitogenically to LPS, but only to LPS preparations obtained by selected extraction methods. These preparations appear to be confined to LPS isolated by mild extraction techniques, such as TCA or butanol. In contrast, those obtained by techniques utilizing phenol were only weakly stimulatory or completely nonstimulatory for spleen cells from the C3H/HeJ. All LPS preparations tested, on the other hand, were highly stimulatory for cells from another mouse strain, namely the C3H/St. The critical importance of the method of extraction of LPS on its mitogenic activity for C3H/HeJ cells was stressed by experiments in which LPS was prepared from *Escherichia coli* K235 using either of two procedures. In these experiments, phenol-extracted LPS, although mitogenic in the C3H/St, was completely nonstimulatory in the C3H/HeJ; whereas, butanol-extracted LPS was highly stimulatory in both strains of mice. This striking difference was attributed to a destructive effect of phenol on LPS, as demonstrated by the fact that treatment of butanol LPS with phenol resulted in a total loss of its mitogenic activity in the C3H/HeJ, but in only a partial loss in the C3H/St. In general, the mitogenic response observed with selected LPS preparations in the C3H/HeJ was quantitatively lower and more transient than that seen with the C3H/St, although qualitatively these responses appeared to be similar. This was evidenced by the

observation that in both mouse strains LPS was a specific mitogen for B cells, a property which was also attributed in both strains to the same distinct structural region of the LPS molecule, that is lipid A. A preparation of LPS that failed to stimulate B cells from the C3H/HeJ nonetheless had the capacity to block activation of these B cells by a stimulatory preparation of LPS. These results strongly suggest that mitogenic stimulation of B cells by LPS is a function of the structural integrity of both the LPS molecule and putative B-cell receptors for LPS.

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