INFECTION AND IMMUNITY, July 1977, p. 205-214
Vol. 17, No. 1

Printed in U.S.A.

Mild Alkaline Hydrolysis of Lipopolysaccharide Endotoxin Enhances Its Mitogenicity for Murine B Cells

GAIL W. GOODMAN AND BARNET M. SULTZER*

Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Received for publication 13 January 1977

Mild alkaline hydrolysis was found to enhance the mitogenicity of lipopolysaccharide endotoxin for murine B lymphocytes. Alkaline-treated lipopolysaccharide also retained its property as a polyclonal activator. Whereas this treatment reduced the lethality of endotoxin for mice, its toxicity for lymphocytes cultured in the absence of fetal calf serum was increased. Lipid analysis indicated that there were no significant changes in the fatty acids of lipid A, but particle size was significantly reduced, and the material was more homogeneous and soluble than untreated lipopolysaccharide. The relationship of these effects on the structure of lipopolysaccharide endotoxin to the mechanism of B-lymphocyte activation is discussed.

Chemical modification of lipopolysaccharide endotoxin (LPS) has been used in numerous laboratories in attempts to dissociate the toxicity of LPS from its other biological properties (47). One of the first modifications used was alkaline hydrolysis (47), which greatly reduces in vivo lethality (7, 29, 31, 51) and pyrogenicity (31) of LPS while enhancing its tolerogenic effects (3, 7). Alkali treatment also enhances the binding of LPS to erythrocytes (16, 27, 29) and reduces its particle size, as determined by molecular weight and sedimentation velocity (27, 31, 51), but such treatment does not affect antigenic specificity (29). Also, alkaline hydrolysis cleaves the ester-linked fatty acids from the lipid A (38) moiety, which is generally accepted to be responsible for most, if not all, of the biological properties of LPS.

LPS is a potent mitogen (2, 3, 33), polyclonal activator (PCA) (2,4), immunogen (26, 47), and adjuvant (11, 45). Recent reports from a number of laboratories (2, 3, 11, 20, 21, 33, 36, 45) have indicated that alkali treatment reduced or totally destroyed these properties, presumably through cleavage of the fatty acid ester residues of lipid A. We, therefore, used alkali-treated detoxified LPS (ALPS) in an attempt to make spleen cells in vitro tolerant to the mitogenic effects of untreated LPS, in order to select for subpopulations of cells that respond to other B-cell mitogens. Contrary to our expectations, ALPS retained its mitogenic activity, was a more efficient mitogen than untreated LPS, and was a potent PCA.

A preliminary report of these experiments has been presented [G. W. Goodman and B. M. Sultzer, Fed. Proc., p. 653, Abstr. no. 2465, 1976].)

MATERIALS AND METHODS

Animals. CBA/J and C3H/HeJ mice, originally obtained from Jackson Laboratories, Bar Harbor, Me., were bred in our laboratory. Congenitally athymic "nude" (nu/nu, BALB/c) mice were obtained from stocks maintained in our institution. Mice of both sexes were used for lymphocyte cultures at 2 to 4 months of age. NYA:NYLAR*(Albany) mice were obtained from the New York State Department of Health and used for in vivo toxicity assays.

Mitogens. LPS from *Escherichia coli* O127:B8 and *Salmonella typhosa* O-901 were prepared by the phenol-water method of Westphal et al. (53). Concanavalin A (ConA) was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., and used at a previously determined optimal concentration of 0.5 μg/ml.

Alkaline hydrolysis. Partial alkaline hydrolysis of LPS was performed by dissolving 50 mg of LPS in 3 ml of 0.25 N NaOH and heating the solution at 56°C for 60 min as described by Neter et al. (29). The treated LPS was precipitated with cold anhydrous ethanol, washed three times with ethanol, dialyzed extensively against nonpyrogenic water, and then lyophilized. *S. typhosa* O-901 LPS and ALPS were used in all reported experiments. Similar results were obtained with *E. coli* O127:B8 LPS.

Lymphocyte cultures. The methods of culture and measurements of deoxyribonucleic acid synthesis by [^{3}H]thymidine uptake have been previously described (49, 50). Spleen cells in the absence of fetal calf serum (FCS) were used at a concentration of 1×10^6 cells/ml, and thymocytes and cortisone-resistant thymocytes were used at 2×10^6 cells/ml. Cortisone-resistant thymocytes were obtained by the method

of Anderson and Blomgren (1). Macrophage-depleted spleen cells were prepared by the carbonyl iron method of Sjöberg et al. (44).

Anti-thy-1 (theta) treatment. CBA/J spleen cells were suspended in RPMI 1640 (GIBCO, Grand Island, N.Y.) at a concentration of 25 × 10^6 cells/ml, and anti-thy-1.2 serum was added to a final concentration of 1:10. The mixture was incubated at 37°C for 30 min, at which time guinea pig complement, adsorbed with CBA/J spleen cells, was added at a final dilution of 1:15, and the incubation continued for an additional 30 min. For mock treatment the same procedure was used but RPMI 1640 was substituted for anti-thy-1.2 serum. The cells were then washed twice with cold RPMI 1640 and suspended to 1 × 10^6 cells/ml. Spleen cell lysis by this treatment was 30 to 44%, whereas mock treatment produced 0 to 4% lysis.

Polyclonal activation. Spleen cell suspensions were prepared without FCS at a concentration of 10 × 10^6 cells/ml. One milliliter of the suspension was incubated in 35-mm plastic petri dishes at 37°C in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2, with or without stimulants, as described by Nilsson et al. (30). Antibody-forming cells were enumerated by a modified hemolytic plaque assay (30) with trinitrophenol (TNP)-conjugated sheep erythrocytes (SRBC). TNP-SRBC were prepared by the method of Rittenberg and Pratt (39).

Chemical analyses. Total nitrogen was determined by the micro-Kjeldahl-ninhydrin method (22), glucosamine by the method of Rondle and Morgan (40), and 2-keto-3-deoxyoctonate by the method of Osborn (32). Fatty acids were determined by hydrolyzing lipid A from *E. coli* LPS with acetic acid (52). Lipid A was extracted into chloroform and dried under reduced pressure. Fatty acids were hydrolyzed and methylated in 0.25 N $NaOCH_3$ as described by Rietschel et al. (38). The resulting methyl esters were dissolved in chloroform for gas chromatography. A coiled stainless-steel column, 6 feet long (ca. 1.83 m), packed with 2.5% Castorwax on Chromosorb G (80 to 100 mesh) (Applied Science Laboratories, State College, Pa.), was used as the support. Nitrogen (25 ml/min) was the carrier gas. The column was run in a Hewlett-Packard 5700A gas chromatograph programmed from 175 to 200°C at a rate of 2°C/min. The final temperature was maintained until all the fatty acids were eluted. All reference standards were obtained from Applied Science Laboratories. The areas under the peaks were quantitated by planimetry.

Toxicity. Toxicity was measured in NYA:NYLAR* mice by the actinomycin D toxicity assay described by Pieroni et al. (34). Actinomycin D (Cosmogen) was obtained from Merck Sharp & Dohme, West Point, Pa. Each vial contained 5 mg of the drug dissolved in nonpyrogenic saline. The drug solution was mixed with the test preparation immediately before testing, and a single intraperitoneal injection was given to each mouse. A three-dose assay was repeated three times with a total of 24 mice per group. The mean lethal dose (LD_{50}) at 48 h was determined by the Reed and Muench method (37).

Electron microscopy. Electron micrographs of LPS and ALPS were taken on a Siemens electron microscope model 1A. LPS and ALPS preparations at a concentration of 10 mg/ml were dried on copper grids and stained with 1% uranyl acetate.

RESULTS

Mitogenicity. A typical dose-response curve of the mitogenic effect of ALPS on CBA/J spleen cells is shown in Fig. 1. The optimal concentration for ALPS was 1 μg/ml, whereas the maximum response with the untreated LPS preparation approximated 100 μg/ml. Furthermore, at high concentrations of ALPS (100 μg/ml), deoxyribonucleic acid synthesis was significantly reduced. This occurred with LPS but at 5 to 10 times higher concentrations (2, 3). Consequently, the bell-shaped dose-response curve for ALPS, which is typical for all mitogens, has a narrower dose-response range than LPS. While there is some variation in the maximal response obtained between 48 and 72 h, in 73% of some 58 trials, 1 to 10 μg of ALPS gave stimulation equal to that of 100 μg of LPS. In the remaining experiments, maximum stimulation by ALPS was only 5 to 7% less than that obtained with LPS.

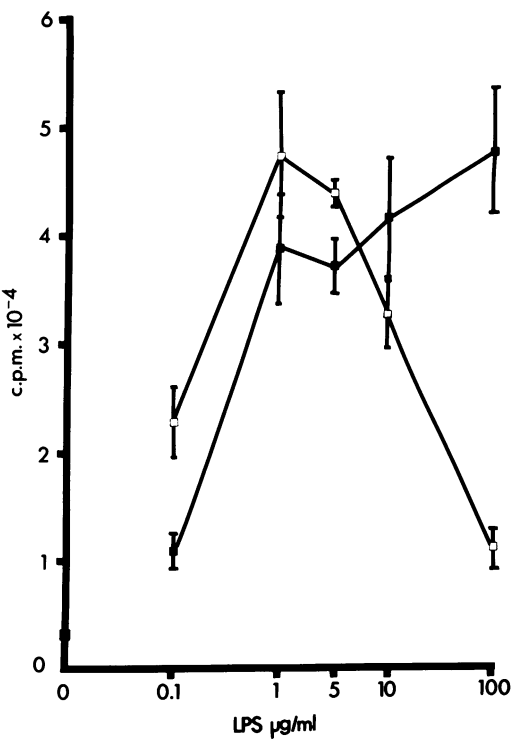

FIG. 1. *Uptake of [^{3}H]thymidine in cultured spleen cells from CBA/J mice after exposure to LPS* (■) *or ALPS* (□) *for 48 h, ± standard error.*

In view of the significant change in the dose response obtained with ALPS, the possibility existed that cells other than B cells might be activated by ALPS. Recently, Kagnoff et al. (23) reported that B lymphocytes from Peyer's patches require the help of T cells to respond to LPS. Therefore, we investigated the response of athymic "nude" (Fig. 2) and anti-thy-1.2-treated (Table 1) spleen cells to LPS and ALPS. Both of these cell populations produced a dose response similar to that seen with CBA/J spleen cells.

The possibility also existed that the mitogenic effect of ALPS might require macrophages. Therefore, macrophage-depleted spleen cell cultures were tested and found to respond to ALPS and LPS in much the same manner as cells cultured with macrophages (Table 2). It should be noted, however, that in cultures depleted of macrophages there was about a 35% reduction in the uptake of [^{3}H]thymidine, and the ALPS dose response was sharper in the absence of macrophages. This may be due to the greater opportunity for interaction of ALPS with lymphocytes (see Discussion). In other experiments (not shown), at 48 h the viability in cultures without macrophages was approximately one-half that seen in cultures with macrophages. This phenomenon also has been found by Chen and Hirsch (10) and by Erb and Feldmann (17). It is unlikely, therefore, either that the greater uptake of [^{3}H]thymidine in the presence of macrophages is due to the proliferation of these cells, or that macrophages are required for ALPS stimulation of B cells per se. Rather, enhanced lymphocyte viability in the presence of macrophages possibly accounts for the somewhat greater incorporation of [^{3}H]-thymidine.

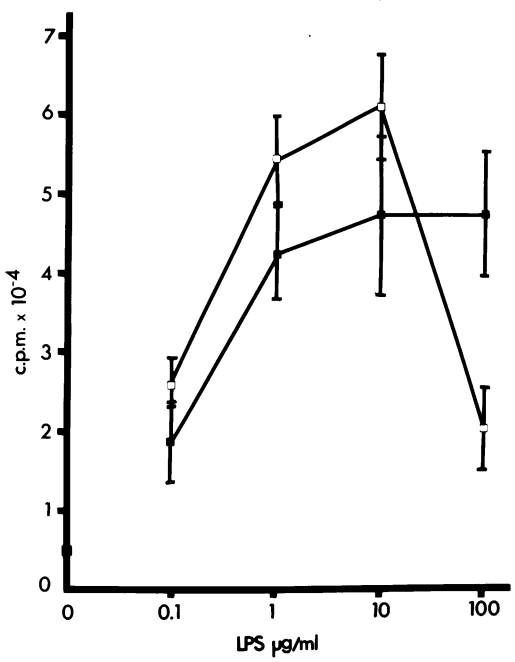

FIG. 2. *Uptake of [^{3}H]thymidine in cultured spleen cells from "nude" mice after exposure to various concentrations of LPS (■) or ALPS (□) for 48 h, ± standard error.*

TABLE 1. *Effect of ALPS on anti-thy-1.2-treated CBA/J spleen cells*

Mitogen	Concn (µg/ml)	Mean [^{3}H]thymidine uptake (cpm) by cultured mouse spleen cells
None		2,706 ± 228[a] (–)[b]
ConA	0.5	3,418 ± 459 (1.3)
LPS	1	36,161 ± 3,535 (13.4)
	10	37,358 ± 3,975 (13.8)
	100	33,705 ± 3,359 (12.5)
ALPS	0.1	17,075 ± 1,556 (6.4)
	1	34,121 ± 3,475 (12.6)
	10	23,121 ± 3,062 (8.6)
ConA (mock-treated)	0.5	21,599 ± 2,526 (8.2)

[a] Standard error of the mean.

[b] Stimulation index: Mean counts per minute of stimulated cultures divided by mean counts per minute of control cultures.

TABLE 2. *Effect of macrophages of lymphocyte proliferation*

Material	Concn (µg/ml)	Mean [^{3}H]thymidine uptake (cpm) by cultured mouse spleen cells
Without macrophages		
None		2,304 ± 495[a] (–)[b]
LPS	1	20,881 ± 4,668 (9.1)
	10	29,496 ± 5,174 (12.8)
	100	34,049 ± 5,412 (14.8)
ALPS	0.1	12,819 ± 2,144 (6.0)
	1	29,569 ± 4,791 (12.8)
	10	17,568 ± 1,976 (7.6)
With macrophages		
None		4,073 ± 186 (–)
LPS	1	24,246 ± 1,289 (6.0)
	10	43,236 ± 1,272 (10.6)
	100	70,224 ± 12,900 (17.2)
ALPS	0.1	13,507 ± 1,065 (3.3)
	1	42,476 ± 2,134 (9.4)
	10	46,459 ± 4,025 (11.4)

[a] Standard error of the mean.

[b] Stimulation index; see footnote *b* of Table 1.

In reports from other laboratories (20, 33, 36, 45), alkaline-hydrolyzed LPS has been tested as a mitogen in cultures containing FCS. This procedure is not routinely used in our laboratory because of the suppressor and enhancer factors found in different lots of FCS (Table 3)

(9, 14, 25, 35). We speculated that the differences between our results and those of other laboratories may be due to the use of FCS. As shown in Table 3, the activity of ALPS in a culture medium supplemented with different lots of FCS (5%) was altered. The optimal concentration of ALPS was shifted to 100 μg/ml, similar to that obtained with LPS in the presence or absence of FCS.

To determine whether T cells could be directly activated by ALPS, thymocytes and cortisone-resistant thymocytes were tested (Table 4). No significant activity was obtained with optimal concentrations of ALPS or LPS, although the cells responded to the T-cell mitogen ConA as expected. In addition, C3H/HeJ spleen cells, which are normally low responders to LPS (13, 50), were not activated by ALPS (Table 4) at concentrations from 0.1 to 100 μg/ml.

Polyclonal activation. We concluded from these results that ALPS is a potent B-cell mitogen. Since all B-cell mitogens are polyclonal activators, ALPS was examined for this property (Fig. 3). In contrast to the mitogen experiments, the activity of ALPS as a PCA closely parallels that seen with LPS at all concentrations when tested against TNP-SRBC.

Toxicity. In view of the steep negative slope of the mitogen dose-response curves with ALPS, the possibility arose that ALPS at high concentrations might be toxic for cultured cells in the absence of FCS.

Spleen cells were cultured without FCS in the usual manner. At 24 or 48 h, 0.05 ml of FCS was added to each culture tube to facilitate recovery of the cells by centrifugation. The su-

TABLE 3. *Effect of different lots of FCS on the mitogenic response of cultured murine spleen cells*

Material	Concn (μg/ml)	Mean uptake of [³H]thymidine (cpm) in FCS lots				
		None	#N43405[a]	#1252[b]	#45562[c]	Pooled[d]
None		1,905 ± 180[e]	31,093 ± 534	5,546 ± 777	7,097 ± 283	6,822 ± 800
LPS	0.1	7,608 ± 496	44,951 ± 2,459	47,108 ± 4,934	34,050 ± 1,528	44,368 ± 1,708
	1	29,798 ± 1,137	47,476 ± 3,461	71,425 ± 8,286	77,196 ± 3,101	65,260 ± 3,515
	10	30,211 ± 1,374	46,271 ± 965	58,053 ± 2,035	44,775 ± 817	57,332 ± 941
	100	72,798 ± 10,253	53,144 ± 3,837	66,255 ± 4,330	59,864 ± 2,263	66,117 ± 208
ALPS	0.1	14,076 ± 1,599	22,006 ± 1,392	19,276 ± 3,202	26,055 ± 3,836	15,993 ± 2,088
	1	39,344 ± 4,374	15,987 ± 1,814	26,969 ± 683	21,026 ± 417	30,447 ± 2,730
	10	32,432 ± 3,711	23,659 ± 2,132	68,911 ± 1,141	54,788 ± 399	69,514 ± 3,531
	100	5,805 ± 518	56,638 ± 5,489	54,776 ± 2,706	68,337 ± 5,332	92,974 ± 10,585

[a] Reheis Chemical Co., Phoenix, Ariz.
[b] Grey Industries, Inc., Fort Lauderdale, Fla.
[c] Flow Laboratories, Inc., Rockville, Md.
[d] Microbiological Associates, Bethesda, Md.
[e] Standard error of the mean.

TABLE 4. *Effect of ALPS on cell populations unresponsive to LPS*

Cells	Material	Concn (μg/ml)	Mean [³H]thymidine uptake (cpm)
CBA/J thymocytes	None		856 ± 160[a] (–)[b]
	ConA	0.5	14,214 ± 3,241 (16.6)
	LPS	100	644 ± 169 (0.8)
	ALPS	1	1,033 ± 251 (1.2)
CBA/J cortisone-resistant thymocytes	None		347 ± 25 (–)
	ConA	0.5	59,364 ± 146 (171.1)
	LPS	100	472 ± 40 (1.4)
	ALPS	1	344 ± 99 (1.0)
C3H/HeJ spleen cells	None		2,160 ± 353 (–)
	LPS	0.1	2,947 ± 295 (1.4)
		1	3,207 ± 232 (1.5)
		10	3,639 ± 639 (1.7)
		100	5,162 ± 375 (2.4)
	ALPS	0.1	2,740 ± 347 (1.3)
		1	2,907 ± 330 (1.3)
		10	4,024 ± 655 (1.9)
		100	2,034 ± 258 (0.9)

[a] Standard error of the mean.
[b] Stimulation index; see footnote *b* of Table 1.

pernatant was removed by aspiration, and the cell pellet was brushed onto glass slides. The dried smears were Wright stained, and blast cells were counted (46). Alternatively, replicate samples of pelleted cells were suspended in 0.1 ml of culture medium, and counts were made for viability by using 0.1% eosin in 3% FCS. In Table 5 decreases in cell viability (27 to 41%) and recovery with ALPS (23 to 30%) are shown as compared with LPS or control cultures at both 24 and 48 h. Blast cells are also decreased in the presence of 100 μg of ALPS. With FCS these differences were not seen at high concentrations of either ALPS and LPS. In contrast,

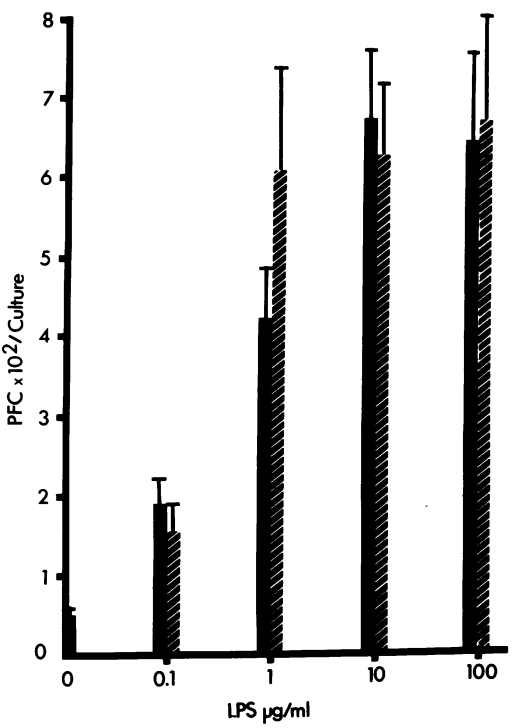

FIG. 3. *Plaque-forming cells (PFC) against TNP-SRBC in CBA/J spleen cell cultures stimulated with LPS (solid bars) or ALPS (hatched bars).*

the mouse toxicity assays showed an LD_{50} of 0.18 μg for LPS and an LD_{50} of 17.6 μg for ALPS. Alkali treatment decreased the in vivo toxicity of LPS by approximately 100-fold.

Chemical analysis. Chemical analysis of the *S. typhosa* LPS indicated 10.9% 2-keto-3-deoxyoctonate, 0.45% nitrogen, and 1.31% glucosamine. ALPS contained 14.6% 2-keto-3-deoxyoctonate, 0.45% nitrogen, an 1.31% glucosamine. Lipid analysis by gas chromatography of the methylated fatty acids revealed the presence of lauric, myristic, palmitic, and β-hydroxymyristic acids amounting to 17.3% of the total fatty acids in LPS and 21.6% in ALPS. In addition, seven unidentified fatty acids were found. These most likely were the unsaturated and odd-numbered fatty acids that have been reported to be present in amounts varying between 20 and 45% by several laboratories (8, 24, 54). We found the unidentified fatty acids in LPS to be 73.7% and 60.3% in ALPS. However, one unidentified fatty acid peak was not detected in ALPS. Finally, LPS contained 6% stearic acid, whereas ALPS contained 18% stearic acid.

Electron micrographs. Because of the physical changes known to occur with alkali treatment of LPS, we examined LPS and ALPS by electron microscopy. Untreated LPS consisted of ribbon-like bilayer strands (arrows) and disks (arrows), characteristic of LPS preparations (Fig. 4), in addition to some amorphous material, as previously described (5, 42). A preparation of ALPS is seen in Fig. 5. These monolayer, disklike particles (arrows) are considerably smaller (approximately 1/20 the size) and more homogenous than the untreated LPS. These results are consistent with findings previously reported (42).

DISCUSSION

Evidence has been presented that ALPS is significantly more efficient as a B-cell mitogen than LPS. Furthermore, this enhanced activity does not appear to be dependent upon accessory

TABLE 5. *In vitro cell viability without FCS*[a]

Material	24 h		48 h		
	% Total cells recovered ± SE[b]	Total viable cells recovered × 10^5 ± SE[b]	% Total cells recovered ± SE[b]	Total viable cells recovered × 10^5 ± SE[b]	% Blast cells
None	78.0 ± 19.0	5.1 ± 1.5	63.0 ± 14.2	2.5 ± 0.8	1.0
LPS (100 μg/ml)	74.7 ± 15.5	4.1 ± 1.1	62.1 ± 13.3	3.2 ± 0.4	26.0
ALPS (100 μg/ml)	57.3 ± 16.9	3.7 ± 1.3	38.0 ± 9.5	1.9 ± 0.6	14.0

[a] With FCS the recovery is between 71 to 73% and the viability is between 5.2 × 10^5 and 5.9 × 10^5 cells for all samples.

[b] Standard error of the mean.

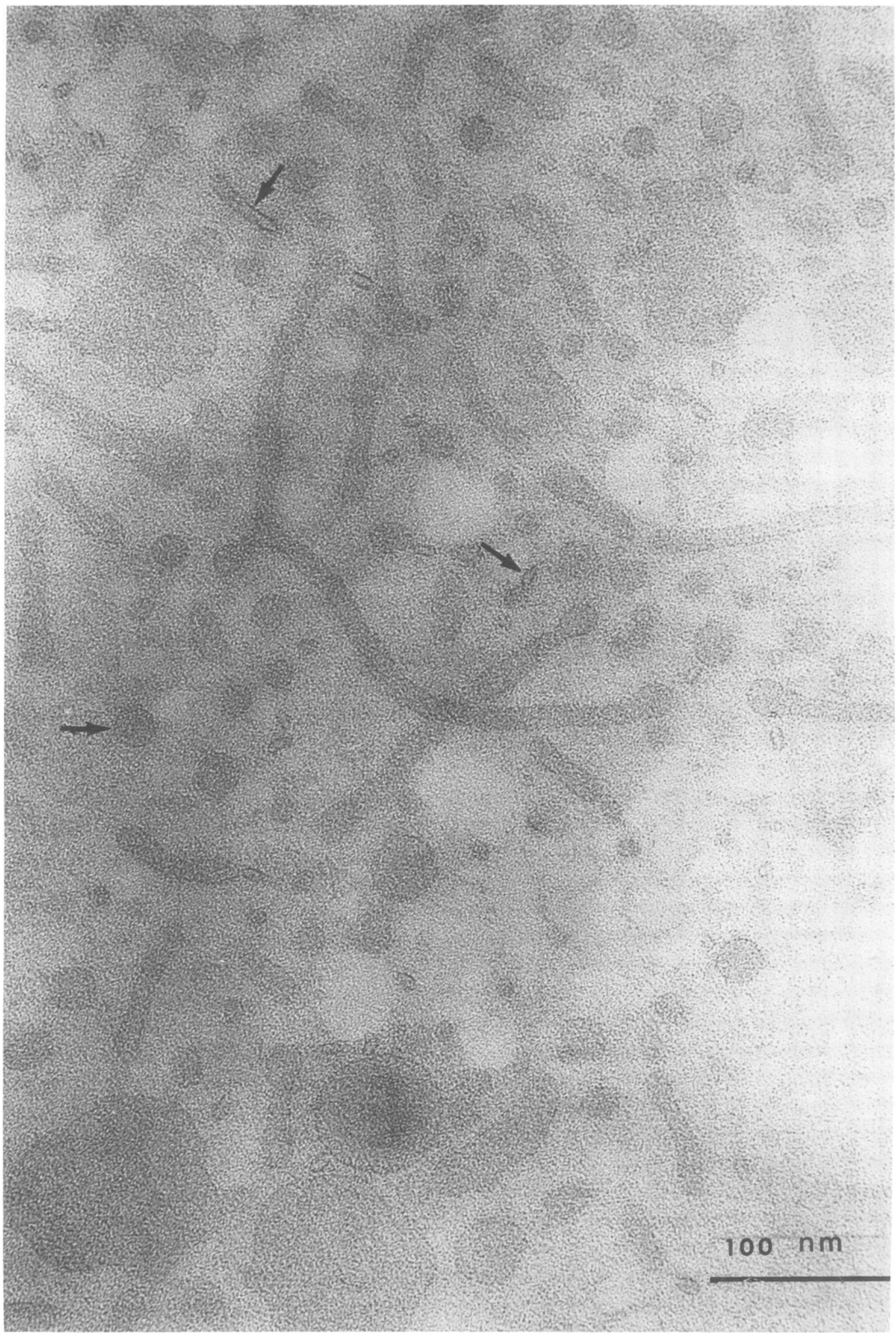

FIG. 4. *S. typhosa O-901 LPS (×280,000).*

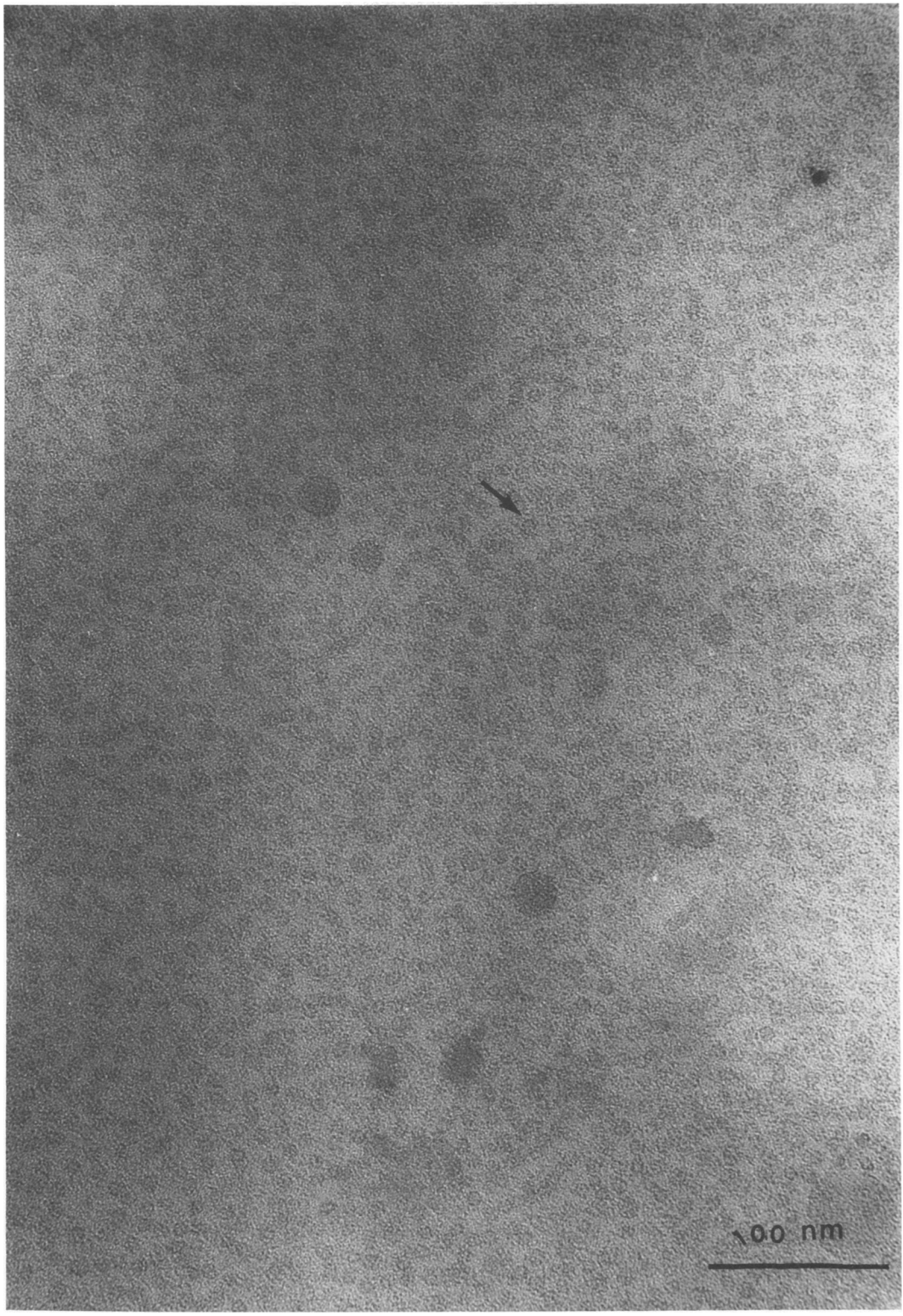

FIG. 5. *S. typhosa O-901 ALPS (×280,000).*

cells, such as T cells or macrophages. On the other hand, as a PCA, ALPS is equivalent in activity to the parent LPS. While it is generally recognized that B-cell mitogens are PCAs, these results with ALPS serve to reiterate that the level of polyclonal activation is not dependent on proliferation and that these two processes are most likely not linked (19, 31).

One of the major questions raised by these results is why any activity is present in the ALPS prepared in our laboratory, since it is generally accepted that alkaline hydrolysis of LPS results in splitting of the fatty acid esters in lipid A with a consequent loss of biological activity. The answer to this question probably can be found in the different methods used in various laboratories.

First, we found that the presence or absence of FCS in the cultures exerts a considerable influence on the response of spleen cells to ALPS, an effect not seen with LPS. In the presence of FCS, the mitogenic effect of ALPS at low concentrations was abrogated. At higher concentrations (100 μg/ml), which were optimally effective with LPS, the stimulatory activity of ALPS returned. Jacobs and Morrison (20), Skidmore et al. (45), and Poe and Michael (36) have reported alkaline-hydrolyzed LPS to be inactive, but all of the culture systems included 5% FCS. A possible explanation for the serum effect on ALPS may reside in the finding that sodium hydroxide-treated LPS forms more stable complexes with proteins than untreated LPS (29). Consequently, the inactivation by FCS at low concentrations of ALPS may be due to the interaction of the ALPS with serum proteins.

The second procedural difference that may have contributed to the results we obtained is the method of alkaline hydrolysis. We hydrolyzed LPS at a concentration of 16.6 mg/ml as originally described by Neter et al. (29). Others have used the same reaction conditions of time, temperature, and alkali concentration, but they suspended the LPS at a lower concentration of 1 mg/ml (20, 45). In addition, alkaline hydrolysis in the presence of ethanol (95%) and the use of higher alkali concentrations, higher temperatures, and longer times of reaction have been described in 12 different protocols (7, 12, 16, 20, 27, 29, 31, 33, 36, 38, 42, 51). All the procedures were more drastic than that originally described by Neter et al. (29), most likely resulting in greater hydrolysis of fatty acid esters. Therefore, it is conceivable that the combination of more rigorous alkaline hydrolysis and the use of culture systems containing FCS has led to inactive preparations. Recently, Morrison has shown that the mitogenicity of alkali-treated LPS can be enhanced when LPS is treated in an ethanolic alkaline solution for 30 min at 37°C. Longer periods of treatment result in reduced and eventually inactive preparations (S. J. Betz and D. C. Morrison, Fed. Proc., p. 1234, 1977).

The method of alkaline hydrolysis we have used is quite mild, as attested to by the lipid analysis obtained. No significant loss of the overall content of fatty acids was obtained in the ALPS. However, one of the major effects achieved with this treatment is physical change in the material. It is more readily soluble, and the particles are considerably smaller and more homogeneous in size and shape than the original phenol-extracted LPS. These changes have also been described by Shands (42) and by Beer et al. (5). Additional evidence of reduced particle size as a result of alkaline treatment has been shown by reduction in molecular weight (51) and in lower sedimentation coefficients (27, 31). One consequence of these physical changes may be the enhanced binding of alkali-treated LPS to erythrocytes as described by Neter et al. (29), Davies et al. (16), Marx et al. (27), and Čižnár and Shands (12). These results also have been observed in our laboratory, as measured by increased hemagglutination titers obtained with ALPS-coated SRBC.

Another effect obtained by the partial alkaline hydrolysis of LPS is the drastic reduction in the in vivo toxicity, which is consistent with previous results (7, 29, 31, 51). The assumption has been that this is due to degradation of lipid A. However, we have reduced the toxicity of the material by about 100-fold without significantly changing the lipid moiety. In striking contrast to the reduced toxicity of ALPS in vivo, ALPS is more toxic than LPS for spleen cells cultured without FCS. In the absence of FCS, cell recovery and viability in cultures stimulated with ALPS are 50 to 75% of that obtained in LPS-stimulated or control cultures. However, in the presence of FCS, cell recovery and viability are approximately equal for all cultures. It is possible that the decreased particle size, increased surface area, and possible change to a monolayer form allow greater interaction of the ALPS with serum factors. The increased interaction of ALPS with serum factors or proteins could serve to reduce the toxicity of ALPS by interfering with the binding of ALPS to cells. It has been shown that serum, lecithin, and cholesterol inhibit the binding of LPS to SRBC and human erythrocytes (28) and that serum can reduce the binding of LPS to rabbit leukocytes by 50% (18). The implication of these experiments is that the serum acts by interfering with the adsorption of LPS to cells rather than

by altering the LPS itself (28). Paradoxically, increased numbers of cells may also alter the toxic effect of ALPS. Thus, in the polyclonal activation experiments where the spleen cell suspensions contained 10 times more lymphocytes, erythrocytes, and macrophages, there was no depression in the number of plaque-forming cells at the ordinarily toxic high concentrations of ALPS.

Shands has suggested that the interaction of LPS with spleen cells is not a passive adsorption, but an active hydrophobic attraction between membrane lipids and lipid A (12, 43). Benedetto et al. (6) have shown that untreated and treated LPS can penetrate artificial lipid bilayers and monolayers and that alkali-treated LPS is 10 times more efficient in this regard. This increased efficiency of penetration is suggested to be a result of the monolayer structure of ALPS (6), and it may be responsible for lysis of erythrocytes obtained in the absence of serum (12).

These results may be analogous to our findings with lymphocytes treated with high concentrations of ALPS in the absence of serum, where total cell recovery was reduced. Cell lysis requires membrane disorganization that could not be achieved through simple adsorption. The decreased cell recovery and viability that we have observed with ALPS indicate that in our system cell lysis, as well as cell death, most likely is occurring. That this occurs with ALPS and not with LPS indicates again that ALPS interacts more efficiently with cell membranes than LPS does. Therefore, at lower concentrations of ALPS where cell viability is not affected, more efficient intercalation with lymphocyte membranes may be interpreted as the cause of enhanced mitogenicity.

Regardless of this greater affinity of ALPS for cell membranes, ALPS still cannot activate the low-responder C3H/HeJ spleen cells. This result indicates that activation of lymphocytes requires more than perturbation of the cell membrane and that triggering is probably dependent upon a secondary or signal receptor (49) as well.

In conclusion, alkali treatment of LPS results in a material that is more readily soluble, is a relatively more homogeneous product, and is a more efficient mitogen than untreated LPS. These characteristics may make ALPS a useful tool in the elucidation of the mechanism of B-lymphocyte activation.

ACKNOWLEDGMENTS

We thank John Butke for technical assistance, Santo Scribani for the electron microscopy, and Michael Howe for his gift of anti-thy-1.2 serum.

LITERATURE CITED

1. **Anderson, B., and H. Blomgren.** 1970. Evidence for a small pool of immunocompetent cells in the mouse thymus. Its role in humoral antibody response against sheep erythrocytes, bovine serum albumin, or albumin and the NIP determinant. Cell. Immunol. 1:362-371.
2. **Anderson, J., F. Melchers, C. Galanos, and O. Luderitz.** 1973. The mitogenic effect of lipopolysaccharide on bone marrow-derived mouse lymphocytes. Lipid A as the mitogenic part of the molecule. J. Exp. Med. 137:943-953.
3. **Anderson, J., G. Moller, and O. Sjöberg.** 1972. Selective induction of DNA synthesis in T and B cells. Cell. Immunol. 4:381-393.
4. **Anderson, J., O. Sjöberg, and G. Moller.** 1972. Induction of immunoglobulin and antibody synthesis *in vitro* by lipopolysaccharides. Eur. J. Immunol. 2:349-353.
5. **Beer, H., A. I. Braude, and C. C. Brinton, Jr.** 1966. A study of particle sizes, shapes and toxicities present in a Boivin-type endotoxic preparation. Ann. N.Y. Acad. Sci. 133:460-475.
6. **Benedetto, D. A., J. W. Shands, Jr., and D. O. Shah.** 1973. The interaction of bacterial lipopolysaccharide with phospholipid bilayers and monolayers. Biochim. Biophys. Acta 298:145-157.
7. **Britton, S.** 1969. Regulation of antibody synthesis against *Escherichia coli* endotoxin. II. Specificity, dose requirements, and duration of paralysis induced in adult mice. Immunology 16:513-526.
8. **Burton, A. J., and H. E. Carter.** 1964. Purification and characterization of the lipid A component of the lipopolysaccharides from *Escherichia coli*. Biochemistry 3:411-418.
9. **Byrd, W.** 1971. Restoration of the immune response to sheep erythrocytes by a serum factor. Nature (London) New Biol. 231:280-282.
10. **Chen, C. N., and J. G. Hirsch.** 1972. Effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells *in vitro*. J. Exp. Med. 136:607-617.
11. **Chiller, J. M., B. J. Skidmore, D. C. Morrison, and W. O. Weigle.** 1973. Relationship of the structure of bacterial lipopolysaccharides to its function in mitogenesis and adjuvanticity. Proc. Natl. Acad. Sci. U.S.A. 70:2129-2133.
12. **Čižnár, I., and J. W. Shands, Jr.** 1971. Effect of alkali-treated lipopolysaccharide on erythrocyte membrane stability. Infect. Immun. 4:362-367.
13. **Coutinho, A., E. Gronowicz, and B. M. Sultzer.** 1975. Genetic control of B cell responses. I. Selective unresponsiveness to lipopolysaccharide. Scand. J. Immunol. 4:139-143.
14. **Coutinho, A., G. Moller, J. Anderson, and W. W. Bullock.** 1973. *In vitro* activation of mouse lymphocytes in serum free medium; effect of T and B cell mitogens on proliferation and antibody synthesis. Eur. J. Immunol. 3:299-306.
15. **Cundy, K. R., and A. Nowotny.** 1968. Comparisons of five toxicity parameters of *Serratia marcesens* endotoxins. Proc. Soc. Exp. Biol. Med. 127:999-1003.
16. **Davies, D. A. L., M. J. Crumpton, I. A. McPherson, and A. M. Hutchison.** 1958. The adsorption of bacterial polysaccharides by erythrocytes. Immunology 2:157-171.
17. **Erb, P., and M. Feldmann.** 1975. Role of macrophages in the generation of T-helper cells. I. The requirement for macrophages in helper cell induction and characteristics of the macrophage T-cell interaction. Cell. Immunol. 19:356-367.
18. **Gimber, P. E., and G. W. Rafter.** 1969. The interaction of *Escherichia coli* endotoxin with leukocytes. Arch.

Biochem. Biophys. 135:14-20.
19. Gronowicz, E., and A. Coutinho. 1976. Heterogeneity of B cells: direct evidence of selective triggering of distinct subpopulations by polyclonal activators. Scand. J. Immunol. 5:55-69.
20. Jacobs, D. M., and D. C. Morrison. 1975. Dissociation between mitogenicity and immunogenicity of TNP lipopolysaccharide, a T independent antigen. J. Exp. Med. 141:1453-1458.
21. Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of a T independent primary anti-hapten response *in vitro* by TNP lipopolysaccharide (TNP-LPS). J. Immunol. 114:360-364.
22. Kabat, E. A., and G. Schiffman. 1962. Immunochemical studies on blood groups. XXVIII. Further studies on the oligosaccharide determinants on blood groups B and BP1 specificity. J. Immunol. 88:782-787.
23. Kagnoff, M. F., P. Billings, and M. Cohn. 1974. Functional characteristics of Peyer's patch lymphoid cells. II. Lipopolysaccharide is thymus dependent. J. Exp. Med. 139:407-413.
24. Kasai, N. 1966. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. Ann. N.Y. Acad. Sci. 133:486-507.
25. Kirchner, H., and J. J. Oppenheim. 1972. Stimulation of chicken lymphocytes in a serum-free medium. Cell. Immunol. 3:695-699.
26. Landy, M., and P. J. Baker. 1966. Cytodynamics of the distinctive immune response produced in regional lymph nodes by *Salmonella* somatic polysaccharide. J. Immunol. 97:670-679.
27. Marx, A., M. Musetescu, M. Sendra, and M. Mihalca. 1968. Relationship between particle size and biological activity of *Salmonella typhimurium* endotoxin. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. 207:313-316.
28. Neter, E., O. Westphal, and O. Lüderitz. 1955. Effects of lecithin, cholesterol, and serum on erythrocyte modification and antibody neutralization by enterobacterial lipopolysaccharides. Proc. Soc. Exp. Biol. Med. 88:339-341.
29. Neter, E., O. Westphal, O. Lüderitz, E. A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocyte modifying, antigenic, toxic and pyrogenic properties. J. Immunol. 76:377-385.
30. Nilsson, B. S., B. M. Sultzer, and W. W. Bullock. 1973. Purified protein derivative of tuberculin induces immunoglobulin production in normal mouse spleen cells. J. Exp. Med. 137:127-139.
31. Niwa, M., K. C. Milner, E. Ribi, and J. A. Rudbach. 1969. Alteration of physical, chemical, and biological properties of endotoxin by treatment with mild alkali. J. Bacteriol. 97:1069-1077.
32. Osborn, M. J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. 50:499-506.
33. Peavy, D. L., J. W. Shands, Jr., W. H. Adler, and R. T. Smith. 1973. Mitogenicity of bacterial endotoxins: characterization of the mitogenic principle. J. Immunol. 111:352-357.
34. Pieroni, R. E., E. J. Broderick, A. Bundeally, and L. Levine. 1970. A simple method for the quantitation of submicrogram amounts of bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 133:790-794.
35. Poe, W. J., and J. A. Michael. 1976. The effect of serum inhibitor on the antigenic and mitogenic responses to *E. coli* bacteria. J. Immunol. 116:1129-1133.
36. Poe, W. J., and J. A. Michael. 1976. Separation of the mitogenic and antigenic responses to bacterial lipopolysaccharide. Immunology 30:241-248.
37. Reed, L., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493-497.
38. Rietschel, E. T., H. Gottert, O. Lüderitz, and O. Westphal. 1972. Nature and linkages of the fatty acids present in the lipid A component of *Salmonella* lipopolysaccharides. Eur. J. Biochem. 28:166-173.
39. Rittenberg, M. B., and K. L. Pratt. 1969. Antitrinitrophenyl (TNP) plaque assay primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132:575-581.
40. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61:586-589.
41. Rudbach, J. A. 1971. Molecular immunogenicity of bacterial lipopolysaccharide antigens: establishing a quantitative system. J. Immunol. 106:993-1001.
42. Shands, J. W., Jr. 1971. Evidence for a bilayer structure in gram-negative lipopolysaccharide: relationship to toxicity. Infect. Immun. 4:167-172.
43. Shands, J. W., Jr. 1973. Affinity of endotoxin for membranes. J. Infect. Dis. 128:S197-S201.
44. Sjöberg, O., J. Anderson, and G. Moller. 1972. Requirement for adherent cells in the primary and secondary immune response *in vitro*. Eur. J. Immunol. 2:123-126.
45. Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O. Weigle. 1975. Immunologic properties of bacterial lipopolysaccharide (LPS): correlation between the mitogenic, adjuvant and immunogenic activities. J. Immunol. 114:770-775.
46. Sultzer, B. M. 1969. Genetic factors in leucocyte responses to endotoxin: further studies in mice. J. Immunol. 103:32-38.
47. Sultzer, B. M. 1971. Chemical modification of endotoxin and inactivation of its biological properties, p. 91-126. *In* S. J. Ajl et al. (ed.), Microbial toxins: a comprehensive treatise, vol. 5. Academic Press Inc., New York.
48. Sultzer, B. M. 1972. Genetic control of host responses to endotoxin. Infect. Immun. 5:107-113.
49. Sultzer, B. M. 1976. A genetic analysis of lymphocyte activation by lipopolysaccharide endotoxin. Infect. Immun. 13:1579-1584.
50. Sultzer, B. M., and B. S. Nilsson. 1972. PPD-tuberculin—a B cell mitogen. Nature (London) New Biol. 240:198-200.
51. Tripodi, D., and A. Nowotny. 1966. Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of *Serratia marcesens*. Ann. N.Y. Acad. Sci. 133:604-621.
52. Westphal, O., and O. Lüderitz. 1954. Chemische Erforschung von Lipopolysaccharide gram-negative Bakterien. Angew. Chem. 66:407-417.
53. Westphal, O., O. Lüderitz, and F. Bister. 1952. Uber die Extraktion von Bacterien mit Phenol-Wasser. Z. Naturforsch. Teil B 7:148-155.
54. Wober, W., and P. Alaupović. 1971. Studies on the protein moiety of endotoxin from gram-negative bacteria. Eur. J. Biochem. 19:340-356.