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LIPOPOLYSACCHARIDE (LPS) REGULATION OF THE IMMUNE RESPONSE: T LYMPHOCYTES FROM NORMAL MICE SUPPRESS MITOGENIC AND IMMUNOGENIC RESPONSES TO LPS[1]

JERRY R. McGHEE, HIROSHI KIYONO, SUZANNE M. MICHALEK, JAMES L. BABB, DAVID L. ROSENSTREICH,[2] AND STEPHAN E. MERGENHAGEN[3]

From The Department of Microbiology, The Comprehensive Cancer Center and The Institute of Dental Research, The University of Alabama in Birmingham, Birmingham, Alabama 35294

The *in vitro* immune responsiveness of cells from germfree (GF) mice was investigated by employing lectins and endotoxin. Splenocytes from GF, when compared with those from conventional (Conv), mice manifested similar responses to the T lymphocyte mitogens, PHA and Con A. When a purified *Escherichia coli* K235 lipopolysaccharide (LPS) was tested, GF splenocytes gave 2- to 3-fold greater stimulation than those observed with Conv spleen cells. This difference in mitogenic responsiveness to LPS was not dependent upon culture conditions or greater numbers of B lymphocytes in GF spleen. Purified lipid A, and to a lesser extent lipid A-associated protein, elicited higher responses with B cells from GF mice. Lower responses to LPS were observed after conventionalization of GF mice with indigenous BALB/c flora. Similar results were obtained when a second LPS effect was measured, anti-TNP PFC responses after TNP-LPS immunization. To determine the nature of the cell(s) involved in this suppression, macrophages (Mϕ) and purified lymphocytes were derived from GF and Conv spleens, and various cell combinations were tested for their mitogenic and immunogenic responsiveness to LPS. No apparent differences in anti-TNP PFC and mitogenic responses could be detected between GF and Conv Mϕ when tested with T and B cells derived from GF mice. However, lower responses were always seen with Conv T and B cells and either GF or Conv Mϕ, suggesting a lymphocyte origin for suppression. Purified T cells from either Conv spleen or Peyer's patches significantly reduced mitogenic and TNP-LPS immune responses in GF spleen cell cultures. The presence of T suppressor cells was further supported by the observation that GF and nude mice exhibited higher responses to TNP-LPS. This evidence taken together suggests that the normal Gram-negative gut flora induces, possibly via lipid A, the production of a T cell population that regulates B cell responses to bacterial endotoxin.

The classical work of Dubos, Schaedler, and their colleagues (1-6) showed that the indigenous Gram-negative flora of the gastrointestinal tract markedly influenced the susceptibility of the host to infection. Their initial studies demonstrated that the Rockefeller Swiss mouse, which had been derived under germfree conditions and maintained, as we now term, specific pathogen-free (SPF)[4] exhibited better growth, greater susceptibility to infection, and marked resistance to lethal effects of bacterial endotoxin (1). Later studies suggested that mice exposed to Gram-negative strains of bacteria exhibited increased susceptibility to the lethal effects of endotoxin (lipopolysaccharide, LPS) (2). Subsequent bacteriologic studies revealed that these SPF, LPS-resistant mice harbored a gut flora of predominantly Gram-positive bacteria (3). Furthermore, the ability to infect these mice with human pathogenic *Escherichia coli* strains was age dependent (4). During the first 2 weeks of lactation, the suckling mice were susceptible to infection, since oral administration of *E. coli* usually resulted in colonization and subsequent death. However, after acquisition of the normal SPF flora, these mice resisted challenge with the human *E. coli* strain (4), clearly suggesting bacterial antagonism. Nevertheless, this mouse colony did eventually attain its own autochthonous *E. coli* flora (5). Although the SPF colony was LPS resistant, the administration of endotoxin to nursing mothers resulted in pronounced depressive effects on the normal growth of their offspring (6).

Proof that germfree (GF) mice are also more resistant to LPS was provided by Jensen *et al.* (7). Although an earlier report by Landy *et al.* (8) suggested that there was no difference between GF and conventional mice to endotoxin, others, utilizing the guinea pig, have shown that the gnotobiotic state does indeed lead, as the work of Dubos had suggested, to LPS resistance (9).

Several studies with GF mice have shown that these animals,

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[2] On sabbatical leave in the Laboratory of Cellular Physiology and Immunology, Rockefeller University, New York from: the Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205.
[3] From the Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, 20205.

[4] Abbreviations used in this paper: Bu-LPS, LPS prepared by butanol-water extraction; Ph-LPS, LPS prepared by phenol-water extraction; LAP, lipid A-associated protein; TNP, trinitrophenyl hapten group; SRC, sheep red cells; nu/nu, athymic, nude mice; nu/+, normal littermates of athymic nude mice; Mϕ, macrophage; GF, germfree BALB/c mice; Conv, conventionally-housed, BALB/c mice; SPF, specific pathogen-free; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GALT, gut-associated lymphoid tissue; PP, Peyer's patch.

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although existing in an almost antigen-free environment, were fully immunocompetent when compared with conventionally-reared (Conv) mice (10–13). Generally, both GF mice and guinea pigs exhibited a delayed response to antigen (10). This could be accounted for by the evidence that GF animals exhibited less lymphoproliferative activity and fewer plasma cells (11). Nevertheless, after antigenic stimulation, GF mice demonstrated increased proliferation of lymphoid cells and of antibody-producing cells (11).

The findings of Schaedler and Dubos (2) have raised the interesting possibility that a normal indigenous flora containing endotoxin-bearing Gram-negative organisms increases the susceptibility of the host to toxic manifestations of LPS. This, coupled with the recent work suggesting that lymphoreticular cells either directly or via their soluble factors mediate the *in vivo* toxic manifestations of LPS (14, 15), make it imperative that lymphoid cells, including macrophages (Mϕ) and T and B lymphocytes from GF and Conv mice be compared in terms of their responsiveness to bacterial endotoxin. The present investigation provides evidence that GF T and B lymphocytes are fully responsive to lectins and LPS, and that GF spleen cells exhibit greater LPS stimulation than is observed in Conv mice. Evidence is provided that Conv mice possess suppressor T lymphocytes that reduce B cell responsiveness to LPS.

MATERIALS AND METHODS

Animals. GF BALB/c mice were bred and maintained under sterile conditions in either rigid plexiglass containers (Germfree Laboratories, Inc., Miami, Fla.) or in Trexler plastic isolators in the Core Facility for Immunocompromised Mice, the Comprehensive Cancer Center at the U.A.B. BALB/c athymic, nude (nu/nu) and their normal littermates (nu/+), original breeding colony obtained from the National Institutes of Health (Bethesda, Md.), were maintained under similar conditions. Conventional BALB/c mice were obtained from Laboratory Supply (Indianapolis, Ind.) or were ex-GF mice that were reared under conventional conditions. Eight- to 12-week-old mice were used in all studies.

Endotoxin preparations. Escherichia coli K235 LPS prepared by a phenol-water extraction (Ph) procedure (16) or by a butanol-water extraction (Bu) method (17) were employed in these studies. Lipid A, derived from *E. coli* 055:B5, and lipid A-associated protein (LAP) obtained from *E. coli* 0111:B4 were the kind gift of Dr. David C. Morrison (Scripps Clinic and Research Foundation, La Jolla, Calif.). TNP-LPS was prepared and utilized as described previously (18).

Mitogens. Phytohemagglutinin (PHA; Burroughs Welcome Company, Greenville, N. C.) and concanavalin A (Con A; Miles Laboratories, Inc., Elkhart, Ind.) were utilized for stimulation of T lymphocytes. The LPS preparations (described above) were employed as B lymphocyte mitogens.

Mitogenesis assay. The mitogenic response of spleen cells to endotoxin and lectins was determined by ^{3}H-TdR uptake as previously described (19). Single spleen cell preparations or purified cells (see below) were resuspended in RPMI 1640 (GIBCO, Grand Island, N. Y.) supplemented with glutamine (2 mM/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and HEPES (see Abbreviations) buffer (pH 7.2, 15 mM). FCS (2%) was added to the cultures in the time-course experiments (Figs. 1 and 2). Cells (8 × 10^5) were cultured in flat-bottom micro trays (Linbro Chemical Co., Hamden, Conn.) and incubated with or without test stimulant for 48 hr, unless otherwise indicated, at 37° C in a humidified atmosphere of 5% CO_2 in air. Cultures were harvested (Mash II, Microbiological Associates, Walkersville, Md.) after a 6-hr pulse of tritiated thymidine (0.5 μCi ^{3}H-TdR; 23 Ci/mM, New England Nuclear, Boston, Mass.).

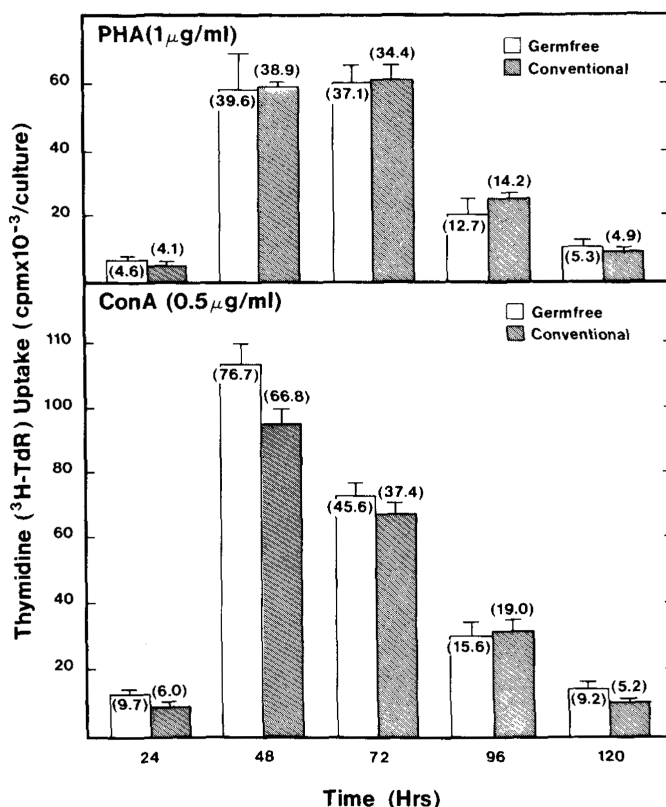

Figure 1. Comparison of mitogenic responses of spleen cells from GF (*open bar*) and Conv (*shaded bar*) BALB/c mice to PHA and Con A. Spleen cells were cultured (8 × 10^5 cells/well) in medium containing FCS (see *Materials and Methods*) for either 24, 48, 72, 96, or 120 hr and incubated with either PHA (1 μg/ml) or Con A (0.5 μg/ml). The concentrations of PHA and Con A employed yielded optimal responses. All cultures were pulsed with 0.5 μCi ^{3}H-TdR during the last 6 hr of incubation. The results are expressed as the arithmetic mean of cpm ^{3}H-TdR uptake/8 × 10^5 cells ± S.E.M. of eight experiments (quadruplicate cultures/experiment). Values in parentheses represent the arithmetic mean of the stimulation ratios (E/C) where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium.

Enumeration of splenic lymphocytes. Fluorescein isothiocyanate-labeled rabbit Fab anti-K and λ light chain sera were used to detect surface immunoglobulin (Ig) on splenic B lymphocytes as described previously (20). Thymus-derived cells were selectively killed by using a monoclonal IgM anti-thy 1.2 sera (21) plus complement (C), a gift provided by Dr. John F. Kearney (U.A.B., Birmingham, Alabama).

Purification of splenic T and B lymphocytes. Mϕ-depleted spleen cell preparations were prepared as described previously (22) by consecutive passage through two Sephadex G-10 columns (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). The effluent cells were used as the source of nonadherent lymphocytes (23).

Purified individual T and B cell populations were prepared from spleen cell suspensions by column fractionation (23). After lysing the erythrocytes with ammonium chloride lysing buffer (10 min, 4°C), the spleen cell preparations were washed, resuspended in medium supplemented with 2% FCS, and passed through glass wool columns to remove the adherent cell population. The nonadherent cell populations were eluted (at room

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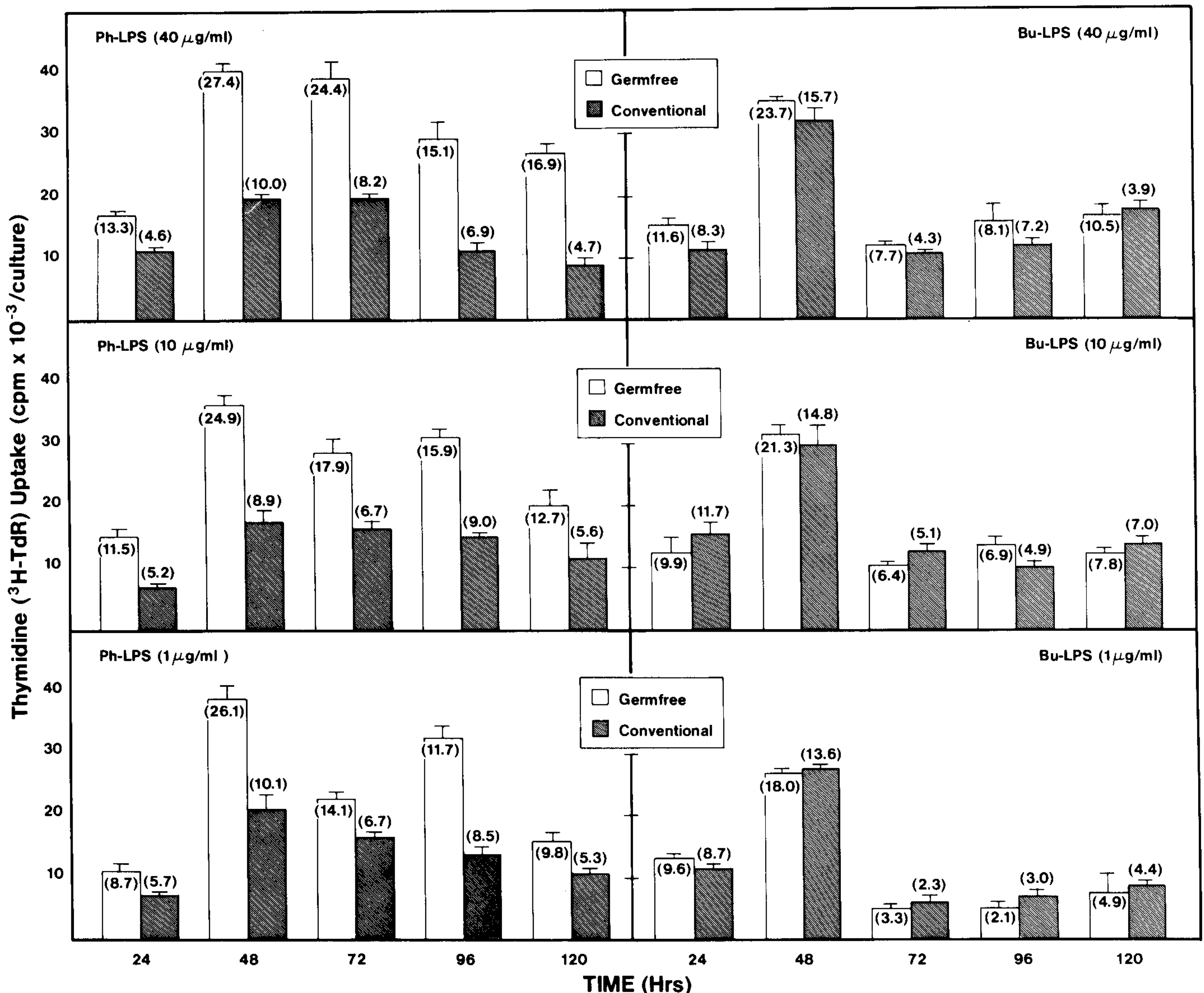

Figure 2. Comparison of mitogenic responses of spleen cells from GF (*open bar*) and Conv (*shaded bar*) BALB/c mice to LPS. Spleen cells were cultured (8×10^5 cells/well) in medium containing FCS (see *Materials and Methods*) for either 24, 48, 72, 96, or 120 hr, and incubated with the indicated concentration of either Ph-LPS (*left panels*) or Bu-LPS (*right panels*). All cultures were pulsed with 0.5 μCi ^{3}H-TdR during the last 6 hr of incubation. The results are expressed as the arithmetic mean of cpm ^{3}H-TdR uptake/8×10^5 cells ± S.E.M. of eight experiments (quadruplicate cultures/experiment). Values in parentheses represent the arithmetic mean of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium.

temperature) with medium supplemented with 2% FCS, and subsequent to centrifugation the nonadherent cells were applied to a nylon wool column according to the method of Julius *et al.* (24). T cell-enriched fractions were obtained by elution of cells from the column with warm medium (37°C), whereas B cell-enriched fractions were obtained by compression of the nylon wool according to the method of Handwerger and Schwartz (25). The T cell fractions were further enriched by treatment with anti-mouse immunoglobulin serum (Meloy Laboratories, Springfield, Va.) and C (26). B cell fractions were further purified by treatment with anti-θ and C.

Purification of splenic Mϕ. Mϕ-enriched populations were prepared by an adherence technique as described previously (23). Single spleen cell preparations were adjusted to 10^7 splenocytes/ml, and after centrifugation the cell pellet was irradiated with 1500 rads. The supernatant was removed, and the cells were resuspended to the original volume in culture medium. Aliquots (0.5 ml) were added to individual culture wells and incubated for 2 hr at room temperature. Nonadherent cells were removed by addition and removal of medium (four times). The adherent cells served as the Mϕ source.

Purification of Peyer's patch T cells. The small intestine of each mouse was aseptically removed from the peritoneal cavity and exposed onto sterile gauze. The Peyer's patches were identified; the serosal side was carefully dissected and placed into medium supplemented with gentamicin (50 μg/ml final concentration). Single cell suspensions were prepared by passage through sterile screen (60 mesh) into medium. Subsequent to washing, the cell suspension was fractionated through a nylon wool column, as described above. The eluted T cell fraction was further enriched by anti-Ig serum and C treatment and served as the source of Peyer's patch T cells.

In vitro immune responses to TNP-LPS. Single spleen cell suspensions were cultured by the method of Mishell and Dutton (27) with modifications (23). Spleen cells (5×10^6) in 0.5 ml complete medium (23, 27) in 16-mm multiwell trays (Linbro

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Chemical Co.) were immunized with 1 μg TNP-LPS, prepared as described previously (18, 28), and incubated (37°C) with rocking in a humidified chamber containing 7% O_2, 10% CO_2, and 83% N_2. The nutritional mixture (0.05 ml) described by Mishell and Dutton (27) was added daily to each culture.

PFC assay. After incubation for 4 days, nonadherent cells were removed from the culture wells, washed, and resuspended at the appropriate dilution for assay. Cultures were assayed in triplicate for anti-TNP and anti-sheep red cell (SRC) PFC utilizing a slide modification (27) of the Jerne and Nordin hemolytic plaque assay. Specific anti-TNP PFC were assessed by utilizing lightly conjugated TNP-SRC (29) when background anti-SRC PFC had been subtracted.

Statistics. Results are expressed as the mean mitogenic or PFC response per culture ± standard error. The significance of difference between means was determined by Student's *t*-test.

RESULTS

Mitogenic responsiveness of spleen cells from GF and Conv mice. The potential of splenic lymphocytes from GF and Conv BALB/c mice to respond to lectins and LPS preparations was evaluated by determining the mitogenic effect of PHA, Con A, and LPS. Spleen cells from GF mice exhibited good responses to the T cell mitogens (Table I), PHA and Con A, which were equal to or greater than the response observed with spleen cells from Conv mice. Significantly higher responses ($p \leq 0.01$) to a Ph-LPS preparation were noted with GF spleen cells. These responses generally were 2- to 3-fold higher than those observed with equal numbers of spleen cells from Conv mice. When an endotoxin preparation that contains LAP (Bu-LPS) was utilized, spleen cells from GF mice responded only slightly higher than the response observed with spleen cells from Conv mice.

Since Conv mice, in contrast to GF mice, are continually exposed to environmental antigens that could affect their lymphoid cells, we next compared the kinetics of the mitogenic responses between GF and Conv splenocytes. In this series of experiments, similar Conv and GF spleen cell cultures were stimulated with lectins or LPS and assayed at daily intervals for 5 days (Figs. 1 and 2). Good PHA responses occurred with both GF and Conv spleen cells after either 48 or 72 hr of culture

TABLE I

Comparison of mitogenic responses of spleen cells from GF and Conv BALB/c mice

Stimulant	GF		Conv	
	Thymidine uptake[a]	E/C[b]	Thymidine uptake	E/C
None	1306 ± 145	1.0	1028 ± 143	1.0
PHA 1 μg/ml	65,676 ± 4428	50.3	48,842 ± 2035	47.5
PHA 0.5 μg/ml	10,749 ± 788	8.2	9118 ± 578	8.9
Con A 0.5 μg/ml	88,042 ± 4043	67.4	86,805 ± 4716	84.4
Con A 0.25 μg/ml	100,903 ± 3540	77.3	83,366 ± 3065	81.1
Ph-LPS 40 μg/ml	39,764 ± 1530	30.4	17,125 ± 2274	16.7
Ph-LPS 10 μg/ml	40,473 ± 1695	31.0	14,770 ± 2268	14.4
Ph-LPS 1 μg/ml	29,293 ± 1954	22.4	13,956 ± 1143	13.6
Bu-LPS 40 μg/ml	29,155 ± 1877	22.3	22,908 ± 1171	22.3
Bu-LPS 10 μg/ml	28,234 ± 1819	21.6	19,854 ± 1609	19.3
Bu-LPS 1 μg/ml	14,010 ± 977	10.7	13,532 ± 809	13.2

[a] Data expressed as cpm ^{3}H-TdR uptake/8 × 10^5 cells. Cells were pulsed for the last 6 hr of a 48 hr culture time. Results represent the arithmetic mean ± S.E.M. of eight experiments (quadruplicate cultures/experiment).

[b] Results represent the arithmetic mean of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium.

TABLE II

Effect of lipid A, LAP and Ph-LPS on mitogenic responses of splenocytes from GF and Conv BALB/c mice

Endotoxin Preparation	GF (E/C)[a]	Conv (E/C)
μg/ml		
Ph-LPS		
40	40.8	20.7
10	46.1	15.7
1	27.4	14.9
Lipid A		
40	10.5	5.8
10	9.6	3.1
1	2.4	1.6
LAP		
20	65.1	51.1
10	66.2	52.0
1	41.9	37.2

[a] Results represent the arithmetic mean of the stimulation ratios of three experiments (quadruplicate cultures/experiment) where E/C = cpm ^{3}H-TdR uptake with endotoxin preparation/cpm ^{3}H-TdR uptake in medium. Cultures (8 × 10^5 cells) were pulsed for the last 6 hr of a 48-hr culture time. Values of controls ranged from 800 to 1200 cpm ^{3}H-TdR/culture.

TABLE III

Comparison of splenocytes from GF and Conv mice for the presence of surface Ig

Spleen Cell Source	% of Cells Bearing Surface Ig
GF	38.7[a]
Conv	39.6

[a] Represents the mean of three separate experiments. In each experiment, spleens were pooled from three to five mice.

(Fig. 1, top panel), whereas Con A elicited maximum stimulation of both GF and Conv spleen cell cultures at 48 hr (Fig. 1, bottom panel). Maximum mitogenic responses were obtained with GF spleen cells stimulated for 2 to 3 days with Ph-LPS (Fig. 2, left panel). These responses were significantly different ($p \leq 0.01$) from those obtained with Conv spleen cells at every concentration of Ph-LPS tested. On the other hand, no difference in responsiveness was observed with spleen cells from either GF or Conv mice treated with Bu-LPS that contained LAP (Fig. 2, right panels). It should be noted that the extraction procedures, either phenol-water or butanol-water, result in LPS preparations that would be expected to differ significantly in M.W. Thus, it is difficult to compare on a molecular basis the results obtained with these two LPS preparations.

In an attempt to obviate this difficulty, the mitogenic effects of purified preparations of lipid A and LAP on spleen cells from GF and Conv mice were compared (Table II). The mitogenic response of GF B splenocytes to either Ph-LPS or lipid A was approximately 2-fold higher than observed with spleen cells from Conv mice. LAP stimulated higher responses in GF spleen cultures and yielded overall greater proliferation than the other endotoxin preparations tested.

To investigate the possibility that greater numbers of B cells occur in GF spleen when compared with Conv mice, spleen cell preparations were assayed for the presence of surface Ig. From the data presented in Table III, it is clear that GF spleens contain similar numbers of B cells expressing Ig when compared with spleens from Conv mice. In separate experiments we have also observed similar numbers of θ-bearing cells when monoclonal anti-θ plus C was utilized to assess cytotoxicity (GF =

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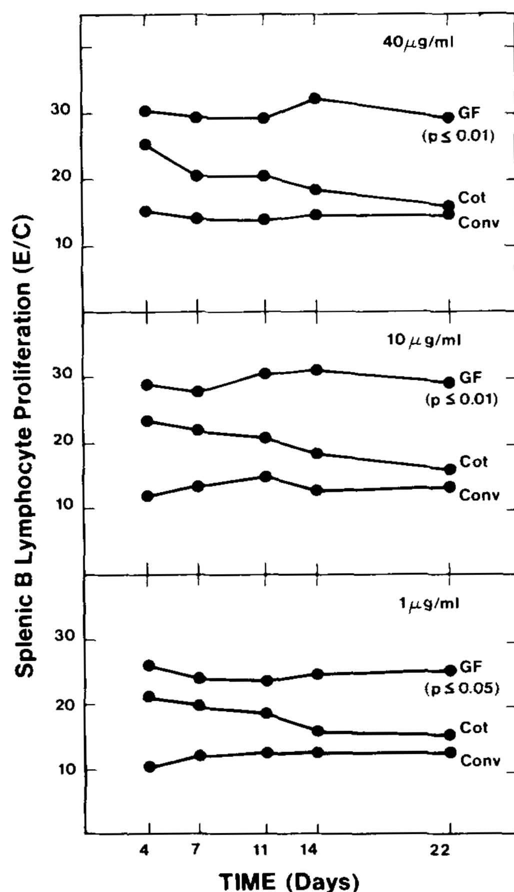

Figure 3. Comparison of mitogenic responses of spleen cells from GF, Conv and conventionalized (Cot) BALB/c mice to Ph-LPS. Spleen cells (8×10^5 cells/culture) were incubated with the indicated concentration of Ph-LPS and pulsed with 0.5 µCi ^{3}H-TdR during the last 6 hr of a 48-hr culture. The time represents day of assay after contamination (conventionalization) of Cot mice. The data is expressed as the arithmetic mean (five experiments; quadruplicate cultures/experiment) of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium (values of controls ranged from 600 to 900 cpm ^{3}H-TdR/culture). The Cot cultures were significantly lower than GF cultures at day 22.

46.5%; Conv = 45.7%).

Effect of conventionalization on B lymphocyte responses to LPS. Since one of the major differences between GF and Conv mice is the presence of an indigenous microbial flora, it was of interest to determine whether infection of GF mice with the indigenous BALB/c microbial flora would alter the pattern of splenic B cell responses to LPS. In these studies, GF mice were removed from the isolators and placed into cages containing feces from Conv BALB/c mice and subsequently maintained under conventional housing conditions. At various times after environmental exposure, spleen cells from these mice (fecal contamination, Cot) were stimulated with Ph-LPS, and the response was compared with that obtained from similarly treated spleen cells from Conv and GF mice (Fig. 3). On day 4 of conventionalization, the responses of Cot spleen cells to all concentrations of Ph-LPS tested were only slightly lower than those responses observed with GF splenocytes. The responses of Cot spleen cells to Ph-LPS subsequently decreased, and by 3 weeks after conventionalization, the splenic response of Cot mice to Ph-LPS was not different (Fig. 3) from that obtained with Conv splenocytes.

In vitro immune responses to TNP-LPS. In order to determine whether the differences obtained in GF and Conv splenic responses are also observed with other LPS effects, we next evaluated immune responses of spleen cell cultures to TNP-LPS (Table IV). GF spleen cells cultures elicited good anti-TNP PFC responses to TNP-LPS (Ph) that were significantly higher ($p \leqq 0.01$) than those obtained with Conv spleen cell cultures. No difference in anti-TNP PFC responses was observed when spleen cell cultures from GF and Conv mice were incubated with TNP-LPS (Bu). Furthermore, spleen cell cultures from either GF or Conv nude mice elicited immune responses to both TNP-LPS preparations that were not different from those responses obtained with spleen cells from GF mice (+/+). These results provided additional evidence that the responsiveness of B cells from Conv mice to LPS was much lower than the responsiveness seen with GF spleen cells.

Characterization of lymphoid cells responsible for suppression of LPS responses. Recent findings of Uchiyama and Jacobs (30, 31) suggested that administration of LPS before immunization suppressed the immune response and that this diminution was probably due to a T suppressor cell population. The following experiments support and extend these observations by demonstrating that mitogenic and immunogenic responses to LPS are modulated by T cells.

In the first series of experiments, the mitogenic responses of splenocytes from ex-GF BALB/c nu/nu or nu/+ mice to lectins and LPS were assessed. From the data presented in Table V, nu/nu spleen cells were significantly ($p \leqq 0.01$) more responsive to the mitogenic effect of Ph-LPS than similar spleen cell cultures from nu/$^+$ littermates. The lack of T cells in these nude mice was evident by the absence, or diminished responsiveness, of spleen cell cultures from these mice to PHA and Con A. Although more than one interpretation of this data is possible (considered below), these results suggested that T cells were involved in the observed suppression of B cell responsiveness to LPS.

In the second series of experiments, increasing numbers of purified splenic T lymphocytes from GF or Conv mice were added to cultures containing purified splenic B cells from GF mice, and the mitogenic response to Ph-LPS was assessed (Fig. 4). Splenic T cells derived from Conv mice suppressed B cell responses to Ph-LPS, whereas T cells from GF mice did not significantly alter the response pattern to Ph-LPS.

To ensure that the splenic B cell from GF mice was not

TABLE IV

Comparison of GF and Conv normal (+/+) and nu/nu splenic responses to TNP-LPS[a]

Spleen Cell Source	Anti-TNP PFC/Culture	
	Ph-LPS	Bu-LPS
GF	1653[b] ± 294	1240 ± 277
Conv	970 ± 70	1490 ± 297
GF Nude	1608 ± 77	1443 ± 46
Conv Nude	1509 ± 88	1414 ± 59

[a] Spleen cells (5×10^6/0.5 ml) from BALB/c mice were cultured with 1 µg/culture of TNP-LPS (Ph) or TNP-LPS (Bu).

[b] Anti-TNP PFC determined on day 4 after incubation; mean PFC ± S.E. of cultures in triplicate/experiment; mean of four experiments. Anti-TNP PFC in control cultures (no antigen) ranged from 75 to 100 and were subtracted from the number of anti-TNP PFC obtained in immunized cultures (see *Materials and Methods*).

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TABLE V

Comparison of splenic mitogenic responses of BALB/c nu/nu and nu/+ mice

Stimulant	Stimulant Concentration	nu/nu		nu/+	
		Thymidine uptake[a]	E/C[b]	Thymidine uptake	E/C
	μg/ml				
None		1,046 ± 22	1.0	851 ± 77	1.0
PHA	1	1,003 ± 103	1.0	50,324 ± 1047	59.1
Con A	0.5	1,121 ± 82	1.0	88,942 ± 1586	104.5
Ph-LPS	40	43,121 ± 1009	41.2	28,251 ± 2747	33.2
Ph-LPS	10	40,095 ± 323	38.3	20,869 ± 1029	24.5
Ph-LPS	1	25,727 ± 667	24.6	14,492 ± 508	17.0
Bu-LPS	40	30,101 ± 751	28.8	32,855 ± 846	38.6
Bu-LPS	10	16,158 ± 548	15.4	19,954 ± 418	23.4
Bu-LPS	1	4,158 ± 248	4.0	7,945 ± 418	9.3

[a] Data expressed as cpm ^{3}H-TdR uptake/8 × 10^5 cells. Cells were pulsed for the last 6 hr of a 48-hr culture time. Results represent the arithmetic mean ± S.E.M. of two experiments (quadruplicate cultures/experiment).

[b] Results represent the arithmetic mean of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium.

peculiar in its responsiveness to Ph-LPS, purified splenic B lymphocytes from GF and Conv mice were tested with an array of Ph-LPS concentrations, and the mitogenic response was measured (Table VI). No significant differences (p≦0.01) were observed in the mitogenic responsiveness of B cells from either GF or Conv mice to Ph-LPS. However, it could be argued that subtle differences exist between GF and Conv B cells that are only manifested in the presence of other lymphoid cell types, e.g., T cells and Mϕ. Therefore, the mitogenic responses of GF and Conv B cells to several concentrations of Ph-LPS were determined in cultures also containing either GF or Conv T cells (Fig. 5). The presence of T cells from GF mice had no effect on the responsiveness of B lymphocytes to Ph-LPS, whereas the presence of T cells from Conv mice significantly (p≦0.01) suppressed the responsiveness of both GF and Conv B cells to Ph-LPS.

Further support that T cells from Conv mice suppress LPS responses was provided when purified splenic B lymphocytes were immunized with TNP-LPS in the presence of purified T cells from either GF or Conv mice (Table VII). The addition of T cells from GF mice to immunized B cell cultures had no effect on the anti-TNP PFC response, compared with the significant (p≦0.01) reduction in the response of cultures containing T cells from Conv mice.

Contribution of Mϕ to LPS suppression of mitogenic and immunogenic responses to LPS. Although the above data clearly demonstrated that T cells from Conv mice suppress LPS responses irrespective of whether B cells were obtained from GF or Conv spleen, it was necessary to evaluate the possible contribution of the third lymphoid element, the Mϕ, in this response. In these studies, splenic adherent cells from either GF or Conv mice were cultured with G-10-passed T and B lymphocytes, and the lymphoproliferative response to Ph-LPS was assessed (Fig. 6). The mitogenic response of GF T and B cell cultures containing either GF or Conv Mϕ was significantly higher (p≦0.01) than similar cultures containing T and B cells from Conv mice. These results demonstrate that splenic Mϕ do not contribute to the observed suppression in responsiveness to LPS. This observation gains additional support by the demonstration that splenic Mϕ, from either GF or Conv mice, do not suppress the anti-TNP PFC responses of cultures of either T

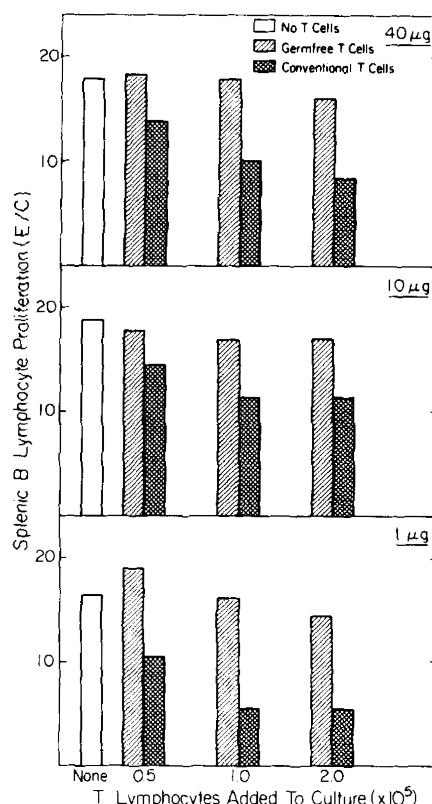

Figure 4. Comparison of the effect of splenic T cells from GF (*single hatched bar*) and Conv (*double hatched bar*) BALB/c mice on the mitogenic response to Ph-LPS of purified splenic B cells from germfree animals. Purified B cells (4 × 10^5/culture) were cultured alone (*open bar*) or in the presence of purified T cells and incubated with the indicated concentration of Ph-LPS. All cultures were pulsed with 0.5 μCi ^{3}H-TdR during the last 6 hr of a 48-hr incubation time. The data is expressed as the arithmetic mean (five experiments; quadruplicate cultures/experiment) of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium (values of controls ranged from 600 to 1000 cpm ^{3}H-TdR/culture). The stimulation ratios of cultures containing germfree T cells were not significantly different (p≦0.01) from controls (no T cells) at all concentrations of Ph-LPS tested. The stimulation ratios of cultures containing conventional T cells were significantly different (p≦0.01) from controls (no T cells) at all concentrations of Ph-LPS tested, except for the cultures containing 0.5 × 10^5 conventional T cells and either 10 or 40 μg Ph-LPS.

TABLE VI

Mitogenic response to Ph-LPS of purified B lymphocytes from GF and Conv BALB/c mice

Stimulant (μg/ml)	GF		Conv	
	Thymidine uptake[a]	E/C[b]	Thymidine uptake	E/C
None	864 ± 35	1.0	950 ± 30	1.0
40	14,812 ± 386	17.1	14,953 ± 447	15.7
20	14,140 ± 182	16.4	14,335 ± 194	15.1
10	13,622 ± 103	15.8	13,731 ± 120	14.5
5	14,868 ± 272	17.2	15,224 ± 177	16.0
1	15,518 ± 973	18.0	14,814 ± 752	15.6
0.5	11,847 ± 70	13.7	12,131 ± 132	12.8

[a] Data expressed as cpm ^{3}H-TdR uptake/2 × 10^5 cells. Cells were pulsed for the last 6 hr of a 48-hr culture time. Results represent the arithmetic mean ± S.E.M. of two experiments (quadruplicate cultures/experiment).

[b] Results represent the arithmetic mean of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium.

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and B cells from GF mice or B cells from nude mice immunized with TNP-LPS (Table VIII).

Origin of T cells responsible for suppression of LPS responses. The evidence demonstrating that splenic T cells suppress LPS responses and that conventionalization of GF mice leads to suppressive effects of LPS responses of spleen cells within 3 weeks imply that lipid A-containing bacteria either induce a T suppressor cell population locally, perhaps in gut-associated lymphoid tissue (GALT), and that these cells could populate the spleen and other tissue, or that the LPS itself actually reaches secondary lymphoid organs, such as the spleen, and effects suppression of responsiveness to endotoxin. Therefore, Peyer's patch and splenic T lymphocytes were prepared from either GF or Conv mice and tested for their ability to suppress TNP-LPS responses of nude mouse spleen cell cultures (Fig. 7). The addition of either splenic or Peyer's patch T

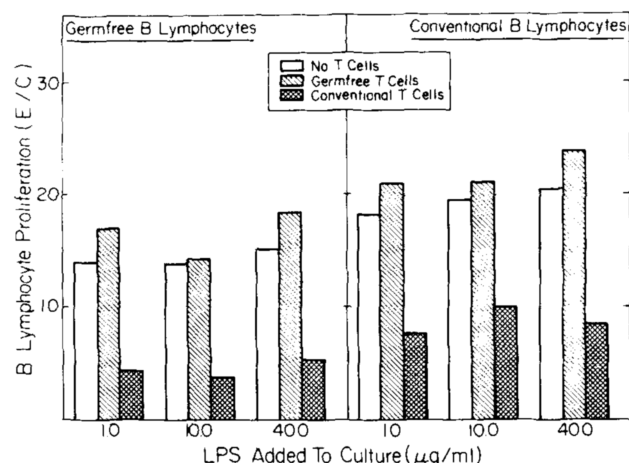

Figure 5. Comparison of the effect of splenic T cells from GF (*single hatched bar*) and Conv (*double hatched bar*) BALB/c mice on the mitogenic response to Ph-LPS of purified splenic B cells from either GF (*left panel*) or Conv (*right panel*) BALB/c mice. Purified B cells (4×10^5/culture) were cultured alone (*open bar*) or in the presence of purified T cells (2×10^5/culture) and incubated with the indicated concentration of Ph-LPS. All cultures were pulsed with 0.5 μCi ^{3}H-TdR during the last 6 hr of a 48-hr incubation time. The data is expressed as the arithmetic mean (five experiments; quadruplicate cultures/experiment) of the stimulation ratios where E/C = cpm ^{3}H-TdR uptake with stimulant/cpm ^{3}H-TdR uptake in medium (values of controls ranged from 600 to 800 cpm ^{3}H-TdR/culture). The stimulation ratios of cultures containing germfree T cells were not significantly different ($p \leqq 0.01$) from controls (no T cells). The stimulation ratios of cultures containing conventional T cells were significantly different ($p \leqq 0.01$) from controls (no T cells) at all concentrations of Ph-LPS tested.

TABLE VII

Effect of splenic T lymphocytes on the response to TNP-LPS of splenic B lymphocytes from GF and Conv BALB/c mice[a]

Source of B Lymphocytes	None	Concentration of T Lymphocyte Added: 1 × 10⁶ GF	1 × 10⁶ Conv	2 × 10⁶ GF	2 × 10⁶ Conv
	(Anti-TNP PFC/culture)[b]				
GF	590 ± 10	625 ± 14	230 ± 40	665 ± 40	110 ± 20
Conv	640 ± 35	617 ± 29	217 ± 29	595 ± 42	220 ± 41

[a] Cultures containing 2×10^6 B lymphocytes/well in 0.5 ml media were incubated with or without TNP-LPS and T lymphocytes and assayed on day 4 for anti-TNP PFC.

[b] Mean anti-TNP PFC ± S.E. per culture. Values represent mean of three experiments, triplicate cultures/experiment.

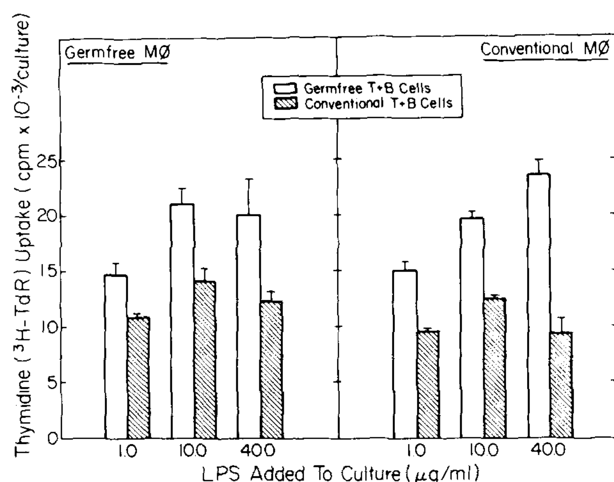

Figure 6. Comparison of germfree and conventional splenic Mϕ affecting the mitogenic response of either germfree T + B cells (*open bar*) or conventional T + B cells (*hatched bar*). Adherent cell populations of Mϕ prepared from irradiated (1500 R) spleen cell preparations (8×10^5 cells/well) were cultured with nonadherent splenic lymphocytes (5×10^5 cells/culture). Cultures were stimulated with Ph-LPS at the indicated concentrations.

TABLE VIII

Effect of splenic macrophages from GF and Conv BALB/c mice on splenic lymphocyte responses[a]

Group	Mϕ[b]	Anti-TNP PFC/Culture
	None	1540 ± 36[c]
GF	GF	1465 ± 48
T + B lymphocytes	Conv	1455 ± 65
	None	881 ± 30
Nude	GF	831 ± 30
B lymphocyte	Conv	931 ± 63

[a] GF splenic lymphocytes (G-10 twice passed) employed at 3×10^6 cells/0.5 ml culture or nude splenic B cells (glass wool passed to remove adherent cells) used at 3×10^6 cells/0.5 ml culture.

[b] GF or Conv spleen cell preparations given 1500 rads of irradiation and allowed to adhere to culture dishes (see *Materials and Methods*).

[c] Anti-TNP PFC determined on day 4 after incubation; mean PFC ± S.E. of cultures in triplicate/experiment; mean of two experiments.

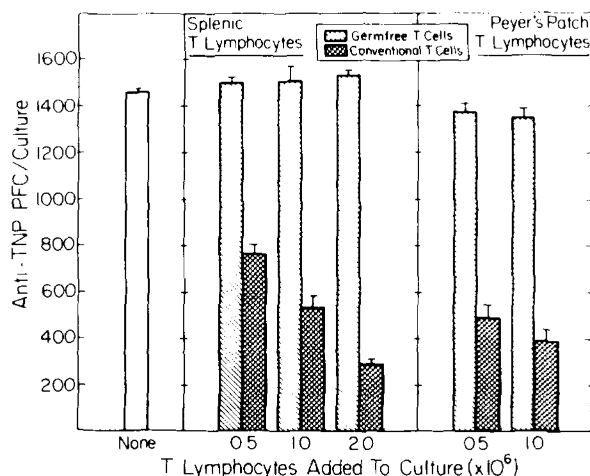

Figure 7. Comparison of germfree (*open bar*) and conventional (*hatched bar*) splenic and PP T lymphocytes affecting the TNP-LPS response of germfree nude mouse spleen cells. The various T lymphocyte preparations were added at the indicated concentrations to 5×10^6/0.5 ml nude splenocytes and immunized with 1 μg TNP-LPS (Ph).

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TABLE IX

Comparison of GF and Conv T cell suppression of nude spleen responses to TNP-LPS[a]

Culture Condition	Anti TNP-PFC/Culture				
	Nude spleen only	GF T cells		Conv T cells	
		Spleen	PP	Spleen	PP
	1204[b] ± 44				
T cells added (1 × 10^6)		1347 ± 127	1190 ± 180	437 ± 34	101 ± 18
T cells (1 × 10^6) + Anti-θ + C		1144 ± 29	1035 ± 39	1146 ± 39	1098 ± 5

[a] GF BALB/c nude spleen cells (5 × 10^6/well) were cultured with or without T lymphocytes and TNP-LPS.

[b] Anti-TNP PFC determined on day 4 after incubation; mean PFC ± S.E. of cultures in triplicate/experiment; mean of three experiments.

cells to TNP-LPS-immunized nude mouse spleen cell cultures significantly ($p \leq 0.01$) reduced the anti-TNP PFC response compared with that obtained with similar cultures containing T cells from GF mice. These results clearly suggested that Peyer's patch T cells from Conv mice suppress TNP-LPS responses. Furthermore, the data presented in Table IX demonstrated that this response was due to direct cell suppression, since prior treatment of these cell preparations with anti-θ and C completely abrogated suppression of responses to TNP-LPS.

DISCUSSION

Although GF mice are undergoing little endogenous immunologic stimulation, the results of this investigation clearly demonstrated that their lymphocytes were fully responsive to mitogenic stimulation and thus were likely to be fully immunocompetent. These findings were in agreement with earlier investigations by others who utilized bacteria (11), soluble protein (10, 11) or heterologous erythrocytes (12) as immunogens. In some instances, delayed and/or prolonged responses were obtained in GF mice (10) that would be expected if there was a lack of suppression, perhaps by T cells. Although GF lymphocytes were fully responsive to mitogens and certain immunogens, it has been suggested that GF mice possess inactive Mϕ (32, 33), which could also account for the characteristics of the responses obtained by others.

In the present studies, significant differences in mitogenic responsiveness to endotoxin (Ph-LPS) were observed. GF spleen cells always manifested enhanced mitogenic responses, which were 2- to 3-fold greater than those seen with Conv splenocytes. This enhanced responsiveness to Ph-LPS was not due to more sensitive cells or to greater numbers (Table III), since differences were always obtained regardless of numbers of cells over the range of Ph-LPS concentrations tested (data not shown). Purified B cells in equal numbers from GF or Conv spleen yielded similar mitogenic responses (Table VI), and significant differences in Ig-positive cells were not observed (Table III). The endotoxin preparation significantly influenced the responses obtained, since Ph-LPS, which probably does not contain LAP and which does not stimulate C3H/HeJ B lymphocytes (18, 23), was most discriminatory when GF and Conv spleen responses were compared. This would imply that these differences were the result of higher lipid A responsiveness in the GF situation, and the data presented here support this contention. However, purified LAP also elicited somewhat better mitogen responses with GF splenocytes. Thus, other regions of the LPS molecule could also account for the observed differences. In this regard, Haas *et al.* (34) reported that the polysaccharide portion of endotoxin could suppress immune responses in the lipid A nonresponder C3H/HeJ mouse. However, it is not clear why Bu-LPS does not elicit higher responses with GF spleen cells, since this preparation would contain two different mitogenic signals, i.e., lipid A and LAP for the B cell. One possible explanation for this would be a physical difference in the different LPS preparations. We know from the work of others (17) that considerable differences in M.W. and other properties are evident when one compares the butanol-water *vs* the phenol-water extracted preparations.

Whatever the mode of induction, albeit lipid A, LAP, or the polysaccharide template of the LPS molecule, Conv mice exhibit a population of suppressor cells that will attenuate the LPS response of B cells to bacterial endotoxin. These cells fractionated as T lymphocytes by standard column procedures and were not affected by anti-Ig and C. The conventionalization studies suggested that significant suppression was present in spleen by 3 weeks after establishment of the gut flora. Further, experiments with purified Peyer's patch (PP) T cells from Conv mice clearly suggested that GALT tissue could serve as reservoirs for this cell type. If indeed the generation of this cell first occurred in GALT from bacterial cell wall stimulation, e.g., LPS, we know from studies of others that GALT T cells home to distant sites, such as lamina propria of the gut (35, 36). They could also account for the presence of these cells in spleen. It is well established that PP T and B cells have a predilection for seeding distant mucosal tissue (37). It might be envisioned that this could represent one means by which the host controls the potential toxic manifestations of LPS when it passes the mucosal barrier (38).

The route of entry of LPS into the host can have a marked effect on the responsiveness of that animal's lymphoid cells. Uchiyama and Jacobs (30, 31) and others (39, 40) have shown that parenteral administration of LPS before antigen resulted in suppression of the immune response, and this suppression was likely T cell derived.

Recent work has clearly suggested that LPS can affect all cells of the lymphoid system. We have provided evidence that both T cells and Mϕ are important in the well-known adjuvant effect of LPS on the immune response (23). Hoffman and Watson (41) have recent evidence that LPS-induced mediators from both Mϕ and T cells augment the immune response. This has been extended to other LPS effects, since Goodman and Weigle (42) recently reported that T cells enhance LPS B cell polyclonal activation. These workers have also implied in their discussion that LPS induces T cells that suppress the polyclonal activation of B cells. Finally, Jacobs (43) has evidence that the T cell lymphokine, T cell-replacing factor, TRF, can act synergistically with LPS toward enhancement of B cell responses to heterologous erythrocytes.

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