SUBCLASS-RESTRICTED IgG POLYCLONAL ANTIBODY PRODUCTION IN MICE INJECTED WITH LIPID A-RICH LIPOPOLYSACCHARIDES*

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Lipopolysaccharides (LPS)[1] from Gram-negative bacteria or the lipid A component isolated from LPS are potent stimulators of murine B lymphocytes in vivo and in vitro. In the absence of antigens, LPS initates proliferation and differentiation of B lymphocytes, leading to the synthesis and secretion of nonspecific IgM polyclonal antibodies (1–3). As a result, mice also develop several types of autoantibodies such as anti-DNA antibodies, rheumatoid factors (RF) and thymocytotoxic antibodies after the injection of LPS (4–8).

Although substantial numbers of IgM antibodies are induced, generally their synthesis is transient and short-lived (7, 9). Some IgG production has been documented as well, but only in an in vitro system (10). Our study has been designed to assess this response further by comparing the capacity of various LPS preparations to induce formation of IgM and IgG polyclonal antibodies, including several types of autoantibodies in adult normal mice and athymic nude (nu/nu) mice. The experiments showed that only lipid A-rich, polysaccharide-free LPS from *Salmonella minnesota* R595 induced long-lasting production of both IgM and IgG in both normal and athymic nude mice. In addition, normal mice made only the IgG2b and IgG3 subclasses in the response to the injection of R595 LPS, but athymic nude mice formed all four subclasses of IgG.

Materials and Methods

Mice. All mice used in this study were 6–10 wk old. C57BL/6, C3H/St, and C3H/HeJ mice were obtained from the mouse breeding colony of the Scripps Clinic and Research Foundation, La Jolla, Calif. Congenitally athymic nude (nu/nu) and heterozygous littermate (nu/+) C57BL/6 mice were kindly provided by Dr. D. E. Parks (Scripps Clinic and Research Foundation). Blood samples were collected by orbital sinus puncture and sera were stored at −20°C until use.

LPS. LPS purified from *S. minnesota* R595 (R595 LPS), *Escherichia coli* 0111:B4, *E. coli* 055:

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[1] *Abbreviations used in this paper:* BBS, borate-buffered saline; BSA, bovine serum albumin; Con A, concanavalin A; DNP, dinitrophenyl; LPS, lipopolysaccharide(s); 2-ME, 2-mercaptoethanol; MEM, minimal essential medium; PFC, plaque-forming cell(s); PII-LPS, fraction II LPS extracted from *Escherichia coli* 0111:B4 with aqueous phenol; R595 LPS, LPS extracted from *Salmonella minneosta* R595; RF, rheumatoid factor; SRBC, sheep erythrocytes; ssDNA, single-stranded DNA; TNP, trinitrophenyl.

B5, *E. coli* K235, and *Salmonella typhimurium* were generously provided by Dr. D. C. Morrison (Department of Microbiology, Emory University School of Medicine, Atlanta, Ga.). *E. coli* 0111:B4 extracted with aqueous phenol (11) was further fractionated by Sepharose 4B column chromatography (12) and fraction II of this preparation (designated PII-LPS) was used in this study because it exhibited more potent activity as polyclonal B cell activators in vivo compared with fraction I LPS (13). R595 LPS extracted as described by Galanos et al. (14) was solubilized by sonication in 0.1% triethylamine and dialyzed against saline. All the LPS preparations were diluted to the desired concentrations with saline and injected intraperitoneally in a final vol of 0.2 ml.

Immunologic Reagents. Murine IgG was purchased from Miles Laboratories, Inc., Elkhart, Ind. Murine myeloma proteins IgG1 (MOPC 21), IgG2a (UPC 10), IgG2b (MOPC 195), IgG3 (Y5606), and IgA (TEPC 15) were obtained from Litton Bionetics, Kensington, Md. Murine myeloma proteins, ABPC 22 (μ,κ) and MOPC 104E ($\mu,\lambda 1$) were prepared as described previously (7).

Anti-murine IgM antibodies were raised in goats repeatedly injected with MOPC 104E and were affinity purified on insolubilized ABPC 22. A polyvalent anti-murine Ig (IgG plus IgM plus IgA) was prepared by immunizing a rabbit with murine Fr II (Miles Laboratories, Inc.). Monospecific rabbit anti-murine IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA antisera were obtained from Litton Bionetics.

Highly polymerized calf thymus DNA (type V) was purchased from Sigma Chemical Co., St. Louis, Mo. Single-stranded DNA (ssDNA) was prepared by heating native DNA (0.5 mg/ml) at 100°C for 10 min, then transferring it immediately to an ice bath. Bovine serum albumin (BSA; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was conjugated with dinitrophenyl (DNP) in a reaction with dinitrobenzenesulfonate, yielding a hapten:protein ratio of 20:1 (15).

Radiolabeling Procedures. Anti-murine IgM was labeled with radioactive iodine (^{125}I) according to the lactoperoxidase procedure of David and Reisfeld (16). ssDNA was labeled with ^{125}I by the method of Commerford (17). DNP_{20}-BSA was iodinated by the chloramine T method (18). ^{125}I-labeled PII-LPS and R595 LPS (19) were kindly provided by Dr. R. J. Ulevitch and Dr. A. R. Johnston (Scripps Clinic and Research Foundation).

Hemolytic Plaque Assay. To measure polyclonal antibody responses, spleens of LPS- or saline-injected mice were removed, placed into chilled minimal essential medium (MEM) and disrupted with a loose-fitting glass homogenizer. After brief sedimentation, cells in the supernates were washed twice in cold MEM and suspended in MEM. The modified Jerne hemolytic plaque-forming cell (PFC) assay (20) was used to detect PFC. Target cells were sheep erythrocytes (SRBC; Colorado Serum Co., Denver, Col.) heavily conjugated with the hapten trinitrophenyl (TNP) by reacting with a solution containing 2,4,6-trinitrobenzene sulfonic acid (ICN Nutritional Biochemicals, Cleveland, Ohio) (21).

Assays of IgM, IgG, and IgA Levels in Sera. Solid-phase radioimmunoassay was performed to assess the serum levels of IgM as described previously (7). 100 μl of 1 μg/ml anti-IgM in borate-buffered saline (BBS), pH 8.4, was used to coat wells of flexible microtiter plates (Cooke Engineering Co., Alexandria, Va.) for 5 h at room temperature. After they were washed three times with BBS, the wells were further coated with 0.5% solution of BSA before 100 μl of a 1:10,000 dilution of test serum samples in BBS that contained 2% BSA was added. The plates were then incubated overnight at 4°C and washed five times with BBS, after which 1 ng of ^{125}I-labeled anti-murine IgM in BBS that contained 2% BSA was added. These plates were incubated another 5 h at 4°C and washed, after which individual wells were cut out for counting. A standard curve was established by using the two murine IgM myeloma proteins ABPC 22 and MOPC 104E.

Serum concentrations of IgG, their subclasses, and IgA were determined by radial immunodiffusion in agar by using monospecific rabbit anti-murine 7S IgG, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, or anti-IgA antisera according to the method of Mancini et al. (22).

Assays of Anti-DNP Antibodies, RF, Anti-ssDNA, and Anti-LPS Antibodies.

ANTI-DNP ASSAY. Serum levels of IgM anti-DNP antibodies were measured as described previously (23) with a radioimmunoassay in which ^{125}I-DNP_{20}-BSA was the antigen. Results are expressed as a percentage of 10 ng ^{125}I-DNP_{20}-BSA precipitated specifically after correction

for nonspecific precipitation in pooled sera of normal mice. To determine IgM and IgG anti-DNP activity in sucrose density gradients, 100 μl of each fraction was incubated with ^{125}I-DNP_{20}-BSA in the presence of 5 μl of pooled normal mouse serum for use as a carrier protein. After incubation at 4°C overnight, goat anti-murine IgM antisera (sufficient to precipitate >95% of the IgM) or rabbit anti-murine Ig antisera (sufficient to precipitate >95% of the IgG) was added as indicated.

IgM RF ASSAY. Serum levels of IgM RF were measured by a solid phase radioimmunoassay with murine IgG used as the antigen. The details of this assay were described previously (7).

ANTI-ssDNA ASSAY. Serum ssDNA-binding activity was determined with a modified Farr DNA binding radioimmunoassay (24). To determine only IgG anti-DNA activity, test sera were incubated with ^{125}I-ssDNA in the presence of 2-mercaptoethanol (2-ME; final concentration 0.1 M), which completely inactivated the IgM anti-DNA activity without affecting IgG anti-DNA activity. The results are expressed as a percentage of 20 ng ^{125}I-ssDNA precipitated specifically. To determine IgM and IgG anti-DNA activity in sucrose density gradients, 100 μl of each fraction was incubated with ^{125}I-ssDNA in the presence of 5 μl of pooled mouse serum.

ANTI-LPS ASSAY. Antibodies against R595 LPS were measured with a radioimmunoassay using ^{125}I-R595 LPS as antigen. 0.1 ml of heat-inactivated serum diluted 1:10 in BBS or 0.1 ml of sucrose gradient fractions was mixed with 0.05 ml of ^{125}I-LPS (10 ng). After incubation at 37°C for 2 h, 0.2 ml of rabbit anti-murine Ig antisera (sufficient to precipitate >95% of the IgM and IgG) was added to precipitate ^{125}I-LPS bound to antibodies. Results are expressed as a percentage of ^{125}I-LPS precipitated specifically after correction for nonspecific precipitation in pooled sera of normal mice.

Sucrose Density-Gradient Ultracentrifugation. 100 μl of sera was layered on a 5–20% linear sucrose density gradient in 0.01 M phosphate-buffered saline, pH 7.0. Samples were centrifuged at 33,000 rpm for 15 h at 4°C with a SW60 rotor in a Beckman L-75 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The positions of IgG and IgM were established by using radioactive markers in gradients divided into 14 fractions.

Lymphocyte Cultures and Measurement of DNA Synthesis. Spleens removed aseptically from test mice were dispersed by gentle teasing with forceps into RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.). Cell aggregates were disrupted by passing the cell suspensions through a 26-gauge needle. Single cell suspensions were washed twice with medium, and the number of viable cells were counted. They were then suspended at a concentration of 10^7 viable nucleated cells/ml in RPMI-1640 medium which was supplemented with 1% L-glutamine (200 mM), 100 U/ml penicillin, and 100 μg/ml streptomycin. 100 μl of spleen cell suspension and 100 μl of various concentrations of LPS or concanavalin A (Con A; Sigma Chemical Co.) in medium were incubated in microculture plates (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) in a 5% CO_2 atmosphere at 37°C for 72 h. 24 h before harvesting, 10 μl of medium that contained 1 μCi of methyl-[^{3}H]thymidine (5 Ci/mmol sp act; Amersham Corp., Arlington Heights, Ill.) was added. Cultures were harvested with a Brandel Cell Harvester (model M24V; Biological Research and Developmental Laboratories, Rockville, Md.)

Results

Long-Lived IgM Polyclonal Antibody Formation After Injection of R595 LPS. The abilities of two different LPS preparations, PII-LPS from *E. coli* 0111:B4 and R595 LPS from *S. minnesota*, to induce polyclonal antibody formation were assessed in vivo by injecting C57BL/6 mice intraperitoneally with the range of doses listed in Table I. 3 d later, polyclonal antibody production in the spleens of these animals was measured by the hemolytic plaque assay with TNP-SRBC as target cells. Injection of 1 μg of R595 LPS or 10 μg of PII-LPS significantly stimulated polyclonal antibody synthesis (Table I). Maximum response was seen with a dose of 10 μg R595 LPS or 50 μg PII-LPS.

The kinetics of polyclonal antibody production in spleens and in sera were compared in mice injected with 50 μg of either R595 LPS or PII-LPS. In spleens, both preparations markedly increased the number of anti-TNP PFC, with the maximum

TABLE I

Polyclonal Anti-TNP PFC Responses in Spleens of Mice Injected with LPS

LPS*	Amount	PFC/10^6 spleen cells	PFC/spleen
	μg		
R595 LPS	0.01	20 ± 8‡	2,020 ± 790
	0.1	28 ± 7	2,525 ± 647
	1	56 ± 6	3,840 ± 1756
	10	293 ± 11	16,851 ± 1276
	50	257 ± 41	20,331 ± 1076
PII-LPS	1	20 ± 2	1,733 ± 230
	10	40 ± 3	2,751 ± 261
	50	254 ± 14	15,127 ± 5479
	100	279 ± 60	19,917 ± 4402
Saline		16 ± 4	1,616 ± 312

* LPS was injected intraperitoneally into C57BL/6 mice and anti-TNP PFC in spleens were measured 3 d after the injection.

‡ Mean of five mice ± 1 SD.

TABLE II

Kinetics of Polyclonal Anti-TNP PFC Responses in Spleens of Mice Injected with LPS

Days after injection	Anti-TNP PFC	
	R595 LPS*	PII-LPS*
1	36 ± 20‡ (1,416 ± 604)§	16 ± 4 (982 ± 230)
3	286 ± 72 (21,107 ± 5,411)	261 ± 71 (15,375 ± 5,077)
5	101 ± 19 (16,328 ± 5,350)	51 ± 14 (6,262 ± 1,313)
8	86 ± 22 (7,484 ± 2,715)	23 ± 3 (1,675 ± 331)
15	80 ± 21 (6,276 ± 2,790)	19 ± 7 (1,350 ± 624)
30	37 ± 13 (4,360 ± 1,134)	12 ± 7 (1,086 ± 547)

* 50 μg of R595 LPS or PII-LPS was injected intraperitoneally into C57BL/6 mice.

‡ Mean anti-TNP PFC/10^6 spleen cells of five mice ± 1 SD. Mean values (±1 SD) of saline-injected control mice were 13 ± 5.

§ Mean anti-TNP PFC/spleen. Mean values (±1 SD) of control mice were 957 ± 494.

measurable on the 3rd d (Table II). On the 8th d, the numbers of anti-TNP PFC in mice injected with PII-LPS were only slightly elevated above values of saline-injected control mice, and after 30 d, spleen cells from these mice were no different from normal spleen cells in this respect. On the other hand, numbers of anti-TNP PFC in spleens of mice injected with R595 LPS remained abnormally high, even after 30 d.

Polyclonal antibody responses to the injection of LPS were assessed in sera by using radioimmunoassays to measure the total levels of IgM and IgM anti-DNP antibody. Both these parameters were increased at 4 d after injection of each type of LPS and peaked on the 8th d (Fig. 1). At peak responsiveness, serum levels of IgM were 10 times higher than saline-injected controls in mice that received R595 LPS and 4 times higher in those that received PII-LPS. In mice injected with PII-LPS, serum concentrations of polyclonal antibodies dropped rapidly and were not significantly different

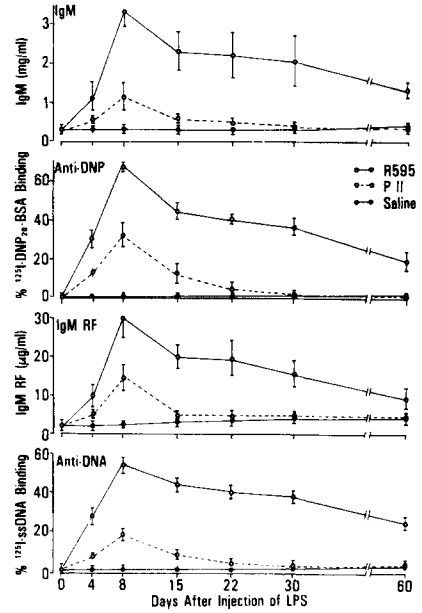

FIG. 1. IgM formation after a single injection of LPS and comparison with the capacity of LPS to induce the production of anti-DNP, IgM-RF, and anti-ssDNA antibodies. 50 μg of R595 LPS or PII-LPS were injected intraperitoneally into C57BL/6 mice on day 0. Each point represents the mean value of seven mice. Vertical bars represent the limits of 1 SD.

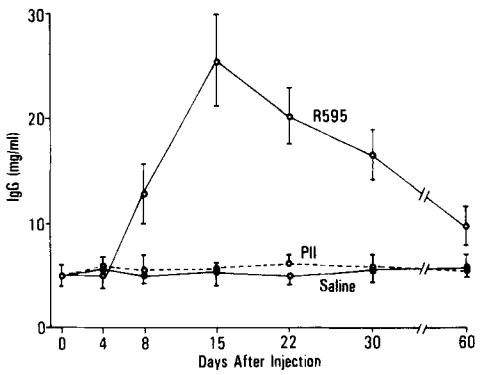

FIG. 2. Serum concentrations of IgG in C57BL/6 mice injected with R595 LPS, PII-LPS, or saline. 50 μg of each LPS were injected intraperitoneally on day 0. Each point represents the mean value of seven mice. Vertical bars represent the limits of 1 SD.

from those of control mice by the 22nd d. However, mice injected with R595 LPS still had high levels of IgM and anti-DNP antibodies even 2 mo after the injection. Serum concentrations of IgM in these mice were approximately seven times higher at 30 d and four times at 60 d than those of control mice.

Because LPS induces the formation of several types of IgM autoantibodies as a result of polyclonal activation of B cells (4–8), the kinetics of two types, IgM RF and anti-ssDNA antibodies, were studied in mice injected with R595 LPS or PII-LPS. Both autoantibody responses were kinetically similar and peaked 8 d after the

injection of each LPS (Fig. 1). Again, R595 LPS, but not PII-LPS, evoked long-lasting production of these autoantibodies similar to the polyclonal antibody response.

IgG Polyclonal Antibody Formation After Injection of R595 LPS. The possible production of IgG polyclonal antibody was investigated in mice after injection of 50 μg R595 LPS or PII-LPS. Serum levels of IgG were measured by radial immunodiffusion using antisera specific for gamma chains. At this dose, PII-LPS never induced significant production of IgG at any time point (Fig. 2). R595 LPS, on the other hand, caused an increased serum IgG beginning day 8 after injection, peaking at levels five times controls on day 15, and persisting up to day 60. In an additional assay, we examined the production of IgA in sera of mice injected with R595 LPS, but found no significant increase at any time during the course of these experiments (data not shown).

To investigate whether IgG induced by LPS was polyclonal antibody, production of 2-ME-resistant IgG anti-ssDNA antibody was followed by using a modified Farr DNA binding radioimmunoassay. 4 d after injection with R595 LPS, test animal sera had only 2-ME-sensitive anti-ssDNA antibodies, but by the 8th d 2-ME-resistant anti-ssDNA antibody activity began to increase significantly until it peaked on day 15, in a time-course similar to that of total IgG production (Fig. 3). At the peak response, ~50% of DNA-binding activity was resistant to the treatment of 2-ME. Sera from mice injected with PII-LPS did not exhibit significant ssDNA-binding activity in the presence of 2-ME.

To confirm further that R595 LPS induced IgG polyclonal antibodies, the immunoglobulin classes of anti-ssDNA and anti-DNP antibodies were analyzed by sucrose density-gradient ultracentrifugation. 8 d after injection, the animals' sera contained a large peak of anti-ssDNA and anti-DNP antibodies in the 19S position (IgM) and a small peak in the 7S position (IgG) (Fig. 4). By day 15, the 7S peaks were

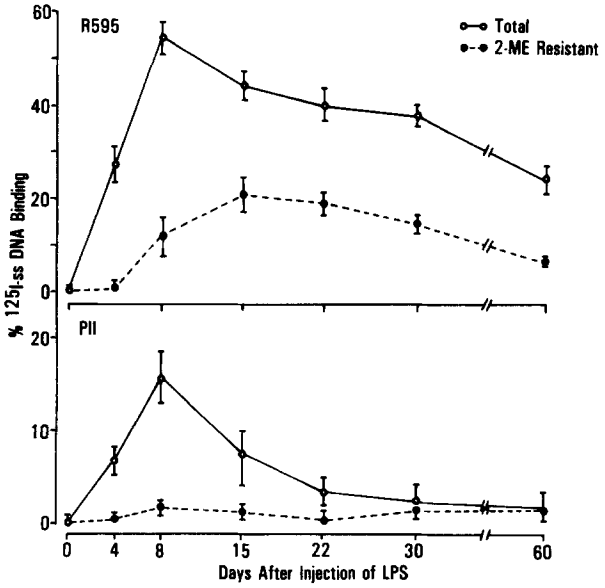

FIG. 3. Serum ssDNA binding-activity in the presence (●) or absence (○) of 2-ME after a single injection of R595 LPS or PII-LPS. 50 μg of each LPS was injected intraperitoneally into C57BL/6 mice on day 0. Each point represents the mean value of seven mice. Vertical bars represent the limits of 1 SD.

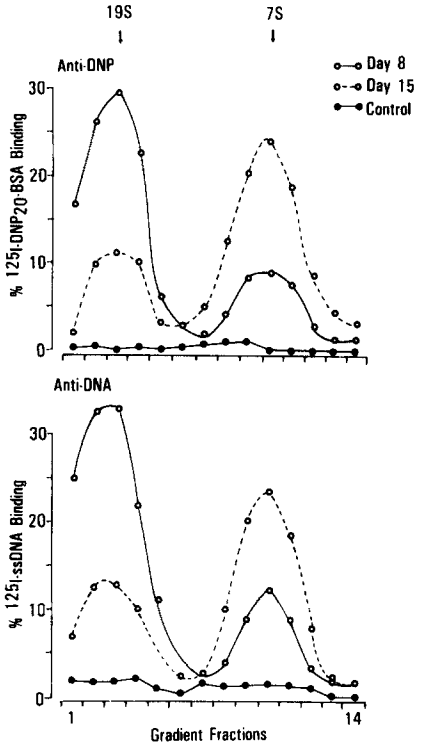

FIG. 4. Ig class analysis of anti-DNP and anti-ssDNA antibodies induced by R595 LPS. Pooled sera from seven C57BL/6 mice were obtained 8 or 15 d after the injection of 50 μg R595 LPS and were fractionated by sucrose density-gradient ultracentrifugation. DNP- and ssDNA-binding activities of each gradient fraction were determined by radioimmunoassays. Arrows indicate the position of markers.

TABLE III

Lack of Response to Injection of R595 LPS in C3H/HeJ Mice

Strains	Treatment	IgM	IgG	Anti-DNA	
				Total	2-ME resistant
		μg/ml	*mg/ml*	%	
C3H/HeJ	R595 LPS*	470 ± 90‡	6.6 ± 1.0	4.6 ± 0.5	0.4 ± 0.4
	Saline	420 ± 10	6.5 ± 1.4	2.4 ± 0.2	0.5 ± 0.2
C3H/St	R595 LPS*	3,370 ± 620	15.0 ± 3.3	24.7 ± 3.7	14.8 ± 2.1
	Saline	410 ± 140	5.3 ± 0.9	2.4 ± 1.0	0.3 ± 0.7

* 50 μg R595 LPS was injected intraperitoneally on day 0. Serum levels of IgM and IgG were determined on day 8 and day 15, respectively. Serum DNA-binding activity was determined on day 15.
‡ Mean of seven mice ± 1 SD.

substantially larger than the 19S ones. The presence of IgG antibodies specific for R595 LPS in these sera or in their gradient fractions was then examined by radioimmunoassays. However, neither the sera nor their gradient fractions exhibited significant anti-R595 LPS activity during the experiments.

The ability of R595 LPS to induce polyclonal IgG antibodies was studied in C3H/

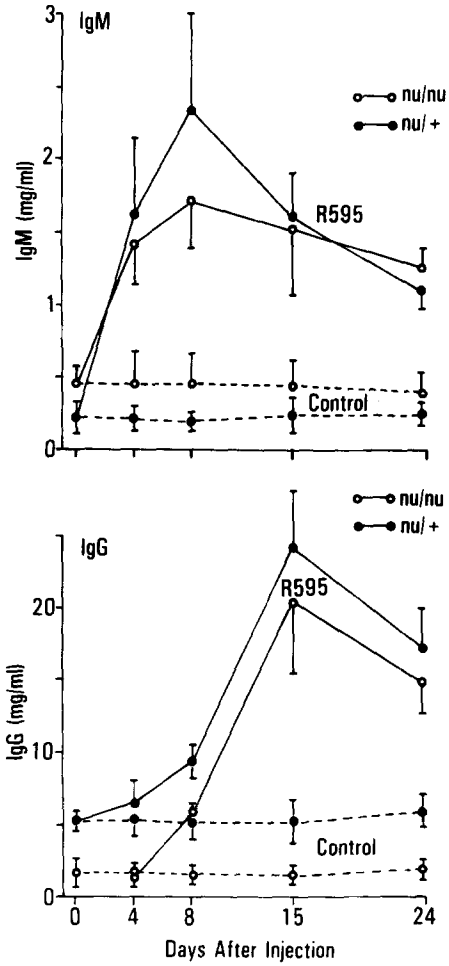

FIG. 5. Serum levels of IgM and IgG in C57BL/6 athymic nude (nu/nu) and heterozygous (nu/+) mice after a single injection of R595 LPS. 50 μg of R595 LPS was injected intraperitoneally on day 0. Each point represents the mean value of seven mice. Vertical bars represent the limits of 1 SD.

HeJ mice because of their known resistance to several biologic effects of LPS (25–29). The LPS-low-responder C3H/HeJ mice and LPS-high-responder C3H/St mice were injected with 50 μg of R595 LPS. As a control, similar groups of mice were injected with saline. The LPS-resistant C3H/HeJ mice showed no increased serum IgM or IgG nor any anti-ssDNA antibodies within 15 d after injection of R595 LPS at the dose used, whereas LPS-responder C3H/St mice produced a significant amount of IgM and IgG polyclonal antibodies in the same situation (Table III).

The effect of LPS on the polyclonal IgG production was studied in athymic nude mice. Homozygous (nu/nu) nude C57BL/6 mice and their heterozygous (nu/+) littermates were injected with 50 μg of R595 LPS. Serum IgG and IgM were similarly increased in both groups on days 4, 8, 15, and 24 (Fig. 5). In addition, nude mice developed as much total and 2-ME-resistant anti-ssDNA antibodies as heterozygous

TABLE IV

Serum DNA-binding Activity in C57BL/6 Nude Mice after Injection of R595 LPS

Genotype	Treatment	Anti-DNA‡	
		Total	2-ME Resistant
		%	
nu/nu	R595 LPS*	26.4 ± 3.8§	12.4 ± 1.9
	Saline	1.7 ± 0.8	−0.1 ± 0.3
nu/+	R595 LPS*	28.6 ± 3.7	10.4 ± 2.6
	Saline	0.2 ± 1.3	−0.3 ± 1.1

* 50 μg R595 LPS was injected intraperitoneally on day 0.
‡ Serum DNA-binding activity was determined on day 15.
§ Mean of seven mice ± 1 SD.

TABLE V

Serum IgM and IgG Levels in C57BL/6 Mice after Injection of LPS

LPS*	Amount	IgM‡	IgG‡
	μg	*μg/ml*	*mg/ml*
R595 LPS	1	500 ± 140§	4.67 ± 0.82
	5	670 ± 200	8.25 ± 1.61
	10	980 ± 460	9.95 ± 2.97
	50	2,260 ± 460	22.25 ± 4.08
	100	2,280 ± 520	23.04 ± 3.96
PII-LPS	50	500 ± 190	4.73 ± 0.29
	100	540 ± 100	4.91 ± 1.17
Saline		100 ± 30	4.77 ± 0.47

* Various doses of R595 LPS or PII-LPS were injected intraperitoneally into C57BL/6 mice on day 0.
‡ Serum levels of IgM and IgG were determined on day 8 and day 15, respectively.
§ Mean of five mice ± 1 SD.

mice (Table IV). Sucrose density-gradient analysis of sera from nude mice confirmed increased amounts of both 19S and 7S anti-ssDNA and anti-DNP antibodies.

The possible differentiation of T cells in nude mice injected with R595 LPS was examined by determining whether spleen cells from these mice responded to the T cell mitogen Con A. Spleen cells from nude mice tested 7 or 14 d after R595 LPS injection and from control nude mice failed to respond to Con A at any dose.

Comparison of Doses and Types of LPS in the Induction of IgG. To establish optimally effective doses of LPS for IgG production, C57BL/6 mice were injected with 1–100 μg/mouse of R595 LPS or PII-LPS. The polyclonal IgG response to injection of R595 LPS was dose-related in that serum IgG concentrations increased significantly after injections >5 μg R595 LPS and were highest in response to >50 μg (Table V). In contrast, PII-LPS, even at a dose of 100 μg, could not induce a detectable IgG polyclonal antibody response. Table V also shows that the dose-response relationship for IgG production was remarkably parallel to that for IgM. In addition, there was a

significant correlation between IgM and IgG in individual samples of sera ($r = 0.890$; $P < 0.001$).

These differences in the capabilities of R595 LPS and PII-LPS to induce IgG polyclonal antibody may be related to their different degrees of localization in lymphoid organs, particularly the spleen. However, we found no clear difference in LPS remaining in spleens at 2, 5, 8, and 15 d after the injection of radiolabeled R595 LPS or PII-LPS; ~0.1% of injected PII-LPS and slightly less R595 LPS was present in the spleens throughout the period of the observation. In addition, radiolabeled and unlabeled R595 LPS induced IgG polyclonal antibody production equivalently.

The ability of various preparations of LPS to induce IgG polyclonal antibodies was tested. C57BL/6 mice were injected intraperitoneally with the same amount (50 μg) of R595 LPS or LPS from *E. coli* 0111:B4, *E. coli* K235, *E. coli* 055:B5, or *S. typhimurium*. Although all the LPS preparations induced significant IgM responses, none except the lipid A-rich and polysaccharide-free R595 LPS provoked substantial IgG polyclonal antibody responses (data not shown). It should also be noted that the concentration of serum IgM induced by R595 LPS was two to four times higher than those induced by other preparations of LPS.

Subclass Restriction of Polyclonal IgG Antibody Induced by R595 LPS. We next determined whether R595 LPS could stimulate the formation of all the IgG subclasses. For this purpose, normal (+/+ or nu/+) and athymic nude (nu/nu) C57BL/6 mice were injected with 50 μg R595 LPS, and total serum IgG for each subclass was determined 8–30 d later. In sera from euthymic C57BL/6 mice, either +/+ or nu/+, R595 LPS greatly increased the amounts of IgG2b and IgG3 with kinetic patterns that were identical to those of total IgG production. At the peak response (on day 15), serum levels of IgG2b and IgG3 were approximately 6 times and 30 times higher, respectively, than those of control mice (Table VI). However, the concentrations of IgG1 and IgG2a did not change. In contrast, R595 LPS induced the formation of all the IgG subclasses in athymic nude (nu/nu) mice. The abnormally low values of IgG1 and IgG2a in the nu/nu saline-injected controls were markedly increased to levels approximating those of saline or LPS-injected nu/+ and +/+ mice.The levels of IgG2b and IgG3 were similar in athymic and euthymic saline-injected controls, and these levels were increased equally by LPS injection in all groups.

TABLE VI

IgG Subclass Produced in Response to R595 LPS in Normal (+/+ or nu/+) and Athymic (nu/nu) C57BL/6 Mice

Genotype	Treatment	IgG1	IgG2a	IgG2b	IgG3
+/+	R595 LPS	1.89 ± 0.44*	2.23 ± 0.29	6.47 ± 1.26	6.24 ± 1.76
+/+	Saline	1.81 ± 0.53	2.33 ± 0.22	1.09 ± 0.04	0.17 ± 0.08
nu/+	R595 LPS	1.71 ± 0.36	2.09 ± 0.19	5.93 ± 1.01	5.80 ± 0.84
nu/+	Saline	1.65 ± 0.60	2.20 ± 0.22	1.14 ± 0.58	0.19 ± 0.11
nu/nu	R595 LPS	1.94 ± 0.50	1.40 ± 0.22	6.16 ± 0.86	5.76 ± 0.91
nu/nu	Saline	<0.05	0.24 ± 0.02	1.53 ± 0.71	0.67 ± 0.26

* Serum concentrations of each IgG subclass in C57BL/6 mice were determined 15 d after the injection of 50 μg R595 LPS. Mean (mg/ml) of seven mice ± 1 SD.

Discussion

In these experiments, we have examined effect of the lipid A-rich, polysaccharide-free R595 LPS on polyclonal antibody formation in mice. R595 LPS had a remarkably greater capability to stimulate B lymphocytes in vivo than the four other preparations of polysaccharide-containing LPS with which it was compared. First, R595 LPS induced accelerated and prolonged production of IgM in various strains of normal mice as well as in athymic nude mice. At the peak response on day 8, their sera had ~4 times more IgM than mice injected with other preparations of LPS and ~20 times more than saline-injected control mice. In addition, mice receiving R595 LPS produced increased amounts of IgM even 2 mo after the injection, but the other LPS preparations induced no activity detectable after 3 wk. Second, R595 LPS stimulated B cells to produce IgG not only in adult normal mice but also in athymic nude mice in vivo. The increased IgG synthesis, which is preceded by increased IgM production, peaked 15 d after injection with levels reaching about five times control.

Several lines of evidence confirm that IgG in addition to IgM is indeed induced by R595 LPS. First, the radial immunodiffusion assay for IgG was completely negative with immunoglobulins of other classes (Materials and Methods). Second, some of the specific antibody activity induced by R595 LPS sediment as 7S IgG in sucrose gradients and is resistant to 2-ME. Finally, the kinetics of IgG induction, measured by radial immunodiffusion or 2-ME resistance of specific antibody activity, was clearly distinguishable from that of IgM formation.

The IgG induced by R595 LPS was not antibody specific for R595 LPS because no anti-R595 LPS antibodies were detectable. Therefore, this IgG most probably formed as a result of polyclonal B cell activation but not from antigenic stimulation. For example, R595 LPS stimulated the production of both 7S anti-DNP antibodies and 7S anti-ssDNA antibodies in parallel with total IgG production.

That athymic C57BL/6 nude mice develop as much IgG as normal mice after the injection of R595 LPS suggests that the production of IgG polyclonal antibodies is not dependent on the presence of T cells. Such T-independent IgG production was indicated in prior studies in vitro and in newborn mice tested in vivo (10, 30). However, because it is known that a few θ-bearing cells may be present in nude mice (31, 32) and because LPS itself may induce precursor cells to differentiate into mature T cells (33, 34), one cannot completely exclude the possible involvement of T lymphocytes in the effect that R595 LPS has on IgG production. The failure of splenic lymphocytes from mice injected with R595 LPS 7 or 14 d previously to respond to the T cell mitogen Con A argues against such a possibility.

It is significant that the stimulation of B cells by R595 LPS in normal mice showed subclass restriction. Serum levels for IgG2b and IgG3 in R595 LPS-injected animals increased to a maximum of 6 and 30 times, respectively, that of control, whereas levels of IgG1, IgG2a, and IgA did not change. This pattern presumably reflects selective stimulation at the precursor-cell level, although we cannot rule out a role for helper or suppressor T cell interactions with subclass-defined B-cell subsets. Recent evidence that IgG3 is the major IgG surface immunoglobulin on murine splenic B lymphocytes (35) as well as the principle subclass represented in immune responses to several carbohydrate antigens (36) further supports the notion of subclass-specific regulatory mechanisms.

In contrast to its restricted effect on normal mice, R595 LPS stimulated the

production of all the IgG subclasses in athymic nude mice. Injection of nu/nu animals with R595 LPS increased their serum levels of IgG1 and IgG2a from markedly diminished levels to concentrations approximating those found in control normals. On the other hand, the levels of IgG2b and IgG3 in nu/nu mice were comparable to those of euthymic controls, and these levels increased after R595 LPS stimulation in parallel fashion in both groups. That nude mice normally had low levels of IgG1 and IgG2a, but not IgG2b and IgG3, as reported here as well as previously (37, 38), and that nude mice with thymus grafts were reconstituted in respect to serum concentrations of IgG2a (30) suggest that the production of IgG1 and IgG2a is more dependent on the presence of functional T cells than that of IgG2b and IgG3. However, our results indicate that T cell help can be replaced with LPS in reconstituting IgG1 and IgG2a to at least normal levels in immunologically deficient athymic nude mice.

Lipid A appears to be essential for LPS to stimulate IgG formation, as suggested by the failure of C3H/HeJ mice, genetic low responders to lipid A (26, 28, 29, 39), to form polyclonal IgG antibody after stimulation by R595 LPS. In addition, R595 LPS has a particularly high content of lipid A: 70% by chemical analysis, as compared with 35% for PII-LPS (D. C. Morrison, personal communication). However, this fact alone cannot explain its unique IgG-stimulating activity, because 5 μg R595 LPS induced a significant IgG response, but 100 μg PII-LPS, which contains ~10 times lipid A, did not. A more likely possibility is that the production of antibodies to polysaccharide O-antigen present in most LPS but not in R595 LPS may subsequently inhibit the full expression of lipid A activity. Alternatively, the lesser degree of solubility of R595 LPS than of other LPS may allow for greater quantities of R595 LPS to localize in tissues, particularly in lymphoid organs; if so, their contact with and stimulation of B cells in tissues might be increased. However, in our related experiments, almost identical amounts of R595 LPS and PII-LPS localized in spleens, and the disappearance rates were no different up to 15 d after injection.

Our data confirm and extend previous studies demonstrating the role of polyclonal B cell activators in the development of autoimmunity (4–8, 40). In contrast to the limited induction of autoimmunity by most preparations of LPS, lipid A-rich, polysaccharide-free R595 LPS can induce a relatively stable state of autoimmunity in normal mice and also stimulate the production of IgG autoantibodies. Although these IgG antibodies are restricted as to subclass, this unique response to the injection of R595 LPS may be, in part, comparable to the spontaneous development of autoantibodies in mice with autoimmune disease as suggested previously (6, 23).

Summary

The effects of five distinct bacterial lipopolysaccharides (LPS) on the induction of polyclonal IgM and IgG antibodies, including polyclonal autoantibody formation, were investigated in several strains of mice. Injections of most LPS preparations that contained polysaccharide transiently induced only IgM polyclonal antibodies. However, LPS from *Salmonella minnesota* R595 (R595 LPS), which had a particularly high content of lipid A but lacked O-antigen polysaccharide, induced a markedly prolonged IgM and IgG polyclonal antibody response in mice, including athymic nude mice, but not in LPS-unresponsive C3H/HeJ mice. Polyclonal IgM and IgG production peaked in sera on day 8 and day 15, respectively, and remained higher than control values 2 mo after the injection. The IgG induced by R595 LPS was strictly restricted

to IgG2b and IgG3 subclasses in normal mice. In contrast, in athymic nude mice which have normally lower levels of IgG1 and IgG2a than normal mice, R595 LPS stimulated the production of all the IgG subclasses and reconstituted serum levels of IgG1 and IgG2a up to, but not higher than, control values of normal mice. These findings suggest that different mechanisms regulate production of each IgG subclass after stimulation with LPS.

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