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# Alteration of the Immunoglobulin G Subclass Responses in Mice to Lipopolysaccharide: Effects of Nonbacterial Proteins and Bacterial Membrane Phospholipids or Outer Membrane Proteins of *Proteus mirabilis*

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The immunoglobulin M (IgM) and the IgG1, $IgG2_{ab}$, and IgG3 subclasses of plaque-forming cells (PFC) specific for lipopolysaccharide (LPS) were measured after immunization of mice with LPS alone and compared with the responses to LPS in combination with nonbacterial proteins and with bacterial membrane phospholipid vesicles or two major outer membrane proteins from *Proteus mirabilis*. The relative numbers of IgG PFC belonging to the IgG1, IgG2, or IgG3 subclasses induced by immunization with LPS alone depended upon the type of LPS administered. Phospholipids and the proteins effected characteristic alterations in not only the strength but also the subclass of the IgG responses to LPS. The results suggest that the hydrophobic-hydrophilic nature or state of aggregation of the preparations plays a role in the induction of IgG1 and IgG2 subclasses of PFC specific for LPS. Complex formation with LPS and adjuvant was apparently necessary to obtain these effects.

Although lipopolysaccharide (LPS) as a type 1 class of thymus-independent antigen stimulates the production of predominantly immunoglobulin M (IgM) antibodies, several reports in the literature over the past few years have established that IgG antibody production in response to LPS, as well as to other thymus-independent antigens, can be detected if sensitive, specific methods are used (1, 8, 18, 23).

In previous publications (7, 21), we reported that outer membrane components of *Proteus mirabilis* have a profound effect in altering both the strength and the character of the antibody-producing cell responses in mice to LPS isolated from this bacterium. In this regard, the immune responses to LPS were altered from a primarily IgM-type response with very little production of IgG to a much stronger response that was mainly IgG in character when LPS was incorporated into bacterial membrane phospholipid vesicles (21) or mixed with a major outer membrane protein from *P. mirabilis* (7).

However, in our earlier investigations, we did not differentiate the IgG responses to LPS into various subclasses. It was therefore of interest to determine which subclasses of IgG-antibody-producing cells are induced in our system by immunization with LPS alone (weak IgG responses) and if the induction of particular IgG subclasses could be altered by mixing the antigen with outer membrane components (strong IgG responses to LPS).

Recently, Slack et al. (23) reported that mice injected with trinitrophenyl (TNP)-LPS produced approximately equal numbers of IgG2 and IgG3 plaque-forming cells (PFC). IgG1 PFC were detectable only in very small numbers. This was apparently typical for responses to type 1 thymus-independent antigens. Kearney (8) has also shown that LPS-stimulated B cells from different lymphoid organs of many mouse strains bear primarily IgG2 and IgG3 on the cell surface.

In the present investigation we have accordingly measured the subclasses of IgG-antibody-producing cells (IgG1, $IgG2_{ab}$, IgG3) specific for LPS induced after immunization of mice with LPS alone or LPS mixed with bacterial membrane components or nonbacterial proteins. The LPSs used were protein-free extracts isolated from *P. mirabilis* and *Escherichia coli*.

## MATERIALS AND METHODS

**Bacterial strains.** *Proteus mirabilis* strains 19 and D52 were from this laboratory (H. H. Martin). *Escherichia coli* F492 (O8:$K27^-$), a capsuleless, smooth strain (20), was obtained from G. Schmidt, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. These organisms were cultivated as previously described (11).

**Extraction and purification of LPS.** LPSs were extracted from whole cells of *P. mirabilis* D52 and *E. coli*

with phenol-water and purified according to Gmeiner (3). Two different types of LPS, LPS I containing short O-polysaccharide chains (one to two repeating units) and LPS II containing long O-polysaccharide chains, can be isolated from *P. mirabilis* (3). To remove contaminating protein from the LPS preparations, the preparations were reextracted with phenol-water containing 1.0% sodium dodecyl sulfate, and LPS was precipitated from the water phase with a mixture of 2 volumes of acetone and 2 volumes of diethyl ether. *P. mirabilis* LPS I and *E. coli* LPS were washed three times with distilled water by ultracentrifugation at 145,000 × *g* for 1 h at room temperature. The sedimented LPSs were resuspended in distilled water and lyophilized. *P. mirabilis* LPS II, which does not sediment upon ultracentrifugation, was dissolved in distilled water and reprecipitated twice with acetone-ether, dissolved in distilled water, and lyophilized. These LPSs were free of protein as determined by amino acid analysis (see below).

**Extraction of phospholipids.** Phospholipids were extracted from whole cells of *P. mirabilis* 19 according to Gmeiner and Martin (5). Composition of the extracts has been described (5, 7, 21).

**Isolation of $M_r$ 39,000 protein.** A major outer membrane protein with a molecular weight of approximately 39,000 ($M_r$ 39,000 protein) was isolated from purified cell walls of *P. mirabilis* 19 according to a method described previously (19). Some batches of the protein isolated and purified in this manner still contained small amounts of LPS detectable by gas-liquid chromatography of fatty acids (see below). The protein was therefore purified further by phenol-water extraction in 0.1% sodium dodecyl sulfate followed by precipitation from the phenol phase with acetone. The precipitated protein was redissolved in a buffer solution containing 1% sodium dodecyl sulfate and subjected to gel filtration in the presence of 0.25% sodium deoxycholate as previously described (19). After extensive dialysis to remove detergent and buffer, the protein was lyophilized. The $M_r$ 39,000 protein forms clear solutions after brief sonication. However, to ensure complete solubilization in buffer solution, the protein was first dissolved in dilute NaOH at pH 9 and immediately neutralized with buffer (19). This protein reacted strongly in hemagglutination assays (titer of 40,000) with antibodies produced against the protein in native form (immunization of rabbits with cell walls).

**Isolation of lipoprotein.** Free lipoprotein with a molecular weight of approximately 7,300 was prepared by extraction of cell walls from *P. mirabilis* 19 with buffer containing sodium dodecyl sulfate at 37°C, followed by column chromatography of the extract on Sephadex G-75/G-50 in the same buffer (4). The free lipoprotein was purified by differential precipitation with acetone (6). The purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis. Details of the procedures will be published elsewhere (J. Gmeiner, manuscript in preparation).

**Commercial proteins.** Bovine serum albumin (BSA) and chymotrypsinogen were obtained from Serva (Heidelberg, Germany). Methylated BSA was purchased from Sigma Chemical Co. (München, Germany).

**Analytical methods.** Protein content was determined by a modification of the Lowry method (10). Amino acid analysis of LPS extracts was performed after hydrolysis in 4.0 N HCl for 16 h at 100°C. Amounts of phospholipids and LPS were determined by gas-liquid chromatography of fatty acids (5).

**Immunization of mice.** A suspension of 0.84 mg of *P. mirabilis* D52 LPS I or LPS II was prepared in 1.0 ml of buffer containing 0.1 M NaCl and 0.02 M Tris-hydrochloride (pH 7.3). For mixtures of LPS with phospholipids or proteins, 1.0 ml of the same amount of LPS in buffer was mixed with either 12.5 μmol (ca. 10 mg) of dried phospholipids or 0.42 mg of lyophilized protein in a conical centrifuge tube. These mixtures (LPS alone, LPS-phospholipids, LPS-protein) were then sonicated for 4 min in an ice bath under a stream of nitrogen by using a Branson model S-125 apparatus (Branson Instruments Co., Danbury, Conn.) at power level 1 with the microtip. The sonicated solutions were diluted 1:6.67 in the above buffer, and 0.2 ml was injected intraperitoneally into white, female, specific-pathogen-free NMRI mice weighing 22 to 25 g (Charles River Wiga, Sulzfeld, Germany). Per injection, dosages of material so prepared were 25 μg of LPS, 25 μg of LPS plus 300 μg of phospholipids, or 25 μg of LPS plus 12.5 μg of protein. For immunization with *E. coli* LPS, amounts of LPS per injection were 12.5 μg instead of 25 μg. A primary injection was given on day 0 and a secondary injection containing the same amount of material on day 14.

To test the responses to sheep erythrocytes (SRBC), mice received a single injection of $4 \times 10^8$ washed SRBC (api-bioMerieux, Nürtingen, Germany) in 0.9% NaCl intraperitoneally.

Separate experiments with the same immunogen always involved separate sets of immunizations performed on different dates.

**Assay of antibody-producing cells.** The IgM- and the IgG-antibody-producing cell responses to LPS were measured in the hemolytic plaque test, using a modification of the microscope slide assay (14) as previously described (7, 21). Briefly, 2.0 ml of a 10% suspension of SRBC sensitized with optimal concentrations of alkali-treated LPS (22) from either *P. mirabilis* D52 or *E. coli* was added to 2.0 ml of lyophilized guinea pig serum (Beringwerke AG, Marburg, Germany) reconstituted 1:2 in Hanks balanced salt solution (14). Then 0.1 ml of this mixture was added to 0.5 ml of 0.6% agarose (Serva, Heidelberg, Germany) in Hanks balanced salt solution in a water bath at 42°C. Immediately thereafter, 0.1 ml of a dilution of spleen cells in Hanks balanced salt solution, pooled from two mice, was added, and the mixture was spread on a microscope slide. Three parallel slides were prepared for each spleen cell dilution. For the measurement of IgG subclass responses, indirect plaques were developed by adding 20 μl of optimal concentrations (1:14 dilution) of specific rabbit anti-mouse IgG1, $IgG2_{ab}$, or IgG3 serum (Nordic Laboratories, Tilburg, The Netherlands) to the test system directly before the addition of spleen cells. The number of IgG PFC was obtained by subtracting the number of direct plaques (IgM) from the number of indirect plaques (IgM + IgG). It should be noted that these antisera did not inhibit the development of IgM plaques.

**Immunoelectrophoresis.** The specificity of the antisera used for developing IgG PFC was tested by immunoelectrophoresis. The buffer used was prepared from a stock solution of 29.43 g of sodium diethylbar-

biturate and 11.71 g of anhydrous sodium acetate (both from E. Merck AG, Darmstadt, Germany) dissolved in a total volume of 1,000 ml with distilled water. Before use, 80 ml of this stock solution was added to 96 ml of distilled water and 26 ml of 0.1 N HCl. The resulting buffer had an ionic strength of 0.15, pH 8.2. Two parts of this buffer were mixed with one part of distilled water for electrophoresis. The gel buffer consisted of one part buffer and two parts distilled water. The agar gel was prepared by dissolving 0.3 g of purified agar (Difco, A. Hedinger KG, Stuttgart, Germany) in 30 ml of gel buffer. The agar gel was poured onto microscope slides (2.5 ml per slide), and wells were filled with 2 μl of normal NMRI mouse serum. Electrophoresis was carried out in a Shandon apparatus (Shandon Labortechnik GmbH, Frankfurt, Germany) at 100 V for 90 min. Precipitin lines were developed against 20 μl of rabbit anti-mouse IgG1, $IgG2_{ab}$, or IgG3 serum used in plaque tests and stained with 2.5 g of Serva Blau (Serva) in a mixture of 225 ml of ethanol, 50 ml of acetic acid, and 225 ml of distilled water.

## RESULTS

**Immunoelectrophoresis of specific IgG subclass antisera.** The specificity of the rabbit anti-mouse IgG1, $IgG2_{ab}$, and IgG3 sera used for the development of indirect plaques was tested by immunoelectrophoresis (Fig. 1). Single precipitin lines in characteristic positions were observed for the IgG1 and the IgG3 antisera. The relatively weak precipitin line for IgG3 reflects the small amount of the immunoglobulin found in normal mouse serum. A double coalescing line was seen in the case of the $IgG2_{ab}$ ($IgG2_a$ + $IgG2_b$) antiserum. These results indicate that according to immunoelectrophoretic analysis, the antisera were specific for the designated IgG subclasses.

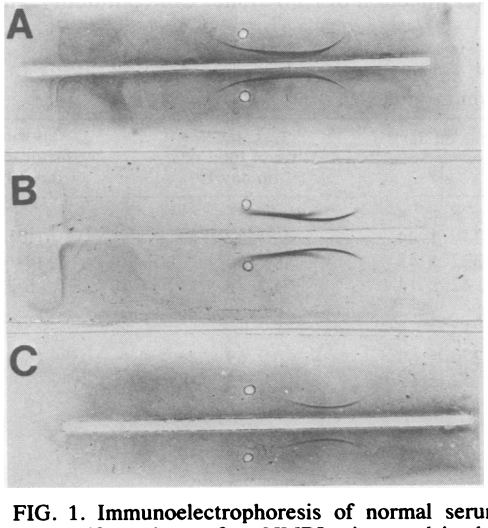


FIG. 1. Immunoelectrophoresis of normal serum from specific-pathogen-free NMRI mice used in this investigation. Precipitin lines were developed against rabbit anti-mouse IgG1 (A), $IgG2_{ab}$ (B), and IgG3 (C) sera. The cathode was at the right, and the anode was at the left.

TABLE 1. IgM and IgG subclass responses in mice to SRBC

| Antibody type | PFC per $10^6$ spleen cells on day 5[a] | IgG subclass (% of total IgG) |
|---|---|---|
| IgM | 1,062 ± 174 | |
| IgG1 | 5,604 ± 1,160 | 81 |
| IgG2 | 1,201 ± 382 | 17 |
| IgG3 | 108 ± 84 | 2 |

[a] Geometric means ± standard errors of PFC obtained after immunization of mice with $4 \times 10^8$ SRBC intraperitoneally on day 0. Responses were measured against SRBC on day 5 after injection. PFC values are the results from five separate experiments.

**Responses in mice to SRBC.** We also tested the specificity of the IgG subclass antisera on the responses to SRBC, a model that has been investigated in the literature (23). The results are presented in Table 1. Immunization of mice with SRBC stimulated predominantly IgG1 PFC (81% of total IgG) and smaller numbers of IgG2 (17%) and IgG3 (2%) cells. These results were similar to those of Slack et al. (23) and further indicate that the IgG antisera were indeed reacting in a subclass-specific manner.

**Responses in mice to *P. mirabilis* D52 LPS I preparations.** Table 2 presents the results of experiments measuring the IgM- and the IgG-antibody-producing cell responses in mice to 25 μg of *P. mirabilis* D52 LPS I alone, 25 μg of LPS mixed with 300 μg of bacterial membrane phospholipid vesicles, 25 μg of LPS mixed with 12.5 μg of $M_r$ 39,000 protein, and 25 μg of LPS mixed with 12.5 μg of lipoprotein isolated from the outer membrane of *P. mirabilis* 19. We used LPS from *P. mirabilis* D52 for these studies to avoid possible cross-reactions of *P. mirabilis* 19 LPS with proteins (2). The dosage of LPS was chosen because it gave minimal but detectable responses. By using a low dose, polyclonal effects were minimized and adjuvant effects could more clearly be seen. The dosages of phospholipids and protein were those which gave pronounced effects in previous studies (7, 21).

Mice received one injection of the above materials on day 0 and a second injection of the same amounts on day 14. Responses were measured on critical days during the course of immunization. Day 4 represents the height of the IgM responses after the first injection, day 14 represents the strongest IgG responses detectable before the second injection, day 18 represents the height of the secondary IgM responses, and day 19 represents the height of the secondary IgG responses (7, 21).

The IgM responses to *P. mirabilis* D52 LPS I

TABLE 2. IgM and IgG subclass responses in mice to *P. mirabilis* D52 LSP I alone and in combination with phospholipids, $M_r$ 39,000 protein, and lipoprotein[a]

| Immunogen[b] | Antibody type | PFC per $10^6$ spleen cells on day[c] | | | | IgG subclass (% of total IgG on day 19) | Adjuvant factor on day 19 |
|---|---|---|---|---|---|---|---|
| | | 4 | 14 | 18 | 19 | | |
| LPS | IgM | 32 ± 4 | 7 ± 1 | 41 ± 7 | 10 ± 1 | | |
| | IgG1 | 0 | 3 ± 1 | 4 ± 2 | 17 ± 3 | 40 | 1 |
| | IgG2 | 0 | 1 ± 1 | 14 ± 3 | 19 ± 5 | 44 | 1 |
| | IgG3 | 0 | 2 ± 1 | 9 ± 3 | 7 ± 1 | 16 | 1 |
| LPS plus phospholipids | IgM | 55 ± 9 | 16 ± 2 | 52 ± 7 | 26 ± 15 | | |
| | IgG1 | 0 | 69 ± 22 | 529 ± 20 | 1,793 ± 507 | 82 | 105 |
| | IgG2 | 0 | 5 ± 2 | 82 ± 11 | 248 ± 12 | 11 | 13 |
| | IgG3 | 0 | 7 ± 1 | 81 ± 18 | 147 ± 21 | 7 | 21 |
| LPS plus $M_r$ 39,000 protein | IgM | 141 ± 5 | 5 ± 1 | 52 ± 25 | 31 ± 5 | | |
| | IgG1 | 0 | 38 ± 3 | 240 ± 35 | 337 ± 75 | 25 | 20 |
| | IgG2 | 0 | 59 ± 1 | 378 ± 71 | 818 ± 133 | 62 | 43 |
| | IgG3 | 0 | 3 ± 3 | 66 ± 22 | 168 ± 49 | 13 | 24 |
| LPS plus lipoprotein | IgM | 49 ± 2 | 5 ± 1 | 41 ± 6 | 12 ± 1 | | |
| | IgG1 | ND[d] | ND | 302 ± 36 | 369 ± 64 | 59 | 22 |
| | IgG2 | ND | ND | 67 ± 38 | 145 ± 21 | 23 | 8 |
| | IgG3 | ND | ND | 34 ± 10 | 113 ± 24 | 18 | 16 |

[a] Responses were measured against *P. mirabilis* D52 LPS I coupled to SRBC.
[b] Dosages per injection were 25 μg of LPS, 300 μg of phospholipids, and 12.5 μg of proteins. Mice received a primary injection on day 0 and a secondary injection on day 14.
[c] Geometric means ± standard errors of PFC. Values are the results of three to five separate experiments.
[d] ND, Not determined.

alone (Table 2) were similar to those determined earlier for the same amount of LPS I from *P. mirabilis* 19 (7, 21). At the height of the secondary IgG responses (day 19), predominantly IgG2 (44% of total IgG) and IgG1 (40%) PFC were detected.

When the same amount of LPS was mixed into bacterial membrane phospholipid vesicles before injection, both the strength and the character of the responses were altered. Regarding the change in IgG isotype, 82% of the IgG PFC measured on day 19 were of the IgG1 subclass compared with only 40% IgG1 cells measured after injection of LPS alone. Concomitantly, the

TABLE 3. IgM and IgG subclass responses in mice to *P. mirabilis* D52 LPS I in combination with BSA, methylated BSA, or chymotrypsinogen[a]

| Immunogen[b] | Antibody type | PFC per $10^6$ spleen cells on day[c] | | IgG subclass (% of total IgG on day 19) | Adjuvant factor on day 19 |
|---|---|---|---|---|---|
| | | 18 | 19 | | |
| LPS plus BSA | IgM | 60 ± 9 | 8 ± 4 | | |
| | IgG1 | 34 ± 19 | 177 ± 44 | 32 | 10 |
| | IgG2 | 171 ± 61 | 322 ± 43 | 60 | 17 |
| | IgG3 | 14 ± 1 | 42 ± 14 | 8 | 6 |
| LPS plus methylated BSA | IgM | 32 ± 11 | 16 ± 6 | | |
| | IgG1 | 124 ± 21 | 270 ± 68 | 69 | 16 |
| | IgG2 | 15 ± 6 | 52 ± 12 | 13 | 3 |
| | IgG3 | 19 ± 3 | 72 ± 24 | 18 | 10 |
| LPS plus chymotrypsinogen | IgM | 44 ± 7 | 11 ± 1 | | |
| | IgG1 | 355 ± 30 | 380 ± 54 | 82 | 22 |
| | IgG2 | 38 ± 12 | 45 ± 9 | 10 | 2 |
| | IgG3 | 6 ± 4 | 37 ± 10 | 8 | 5 |

[a] Responses were measured against *P. mirabilis* D52 LPS I coupled to SRBC.
[b] Dosages per injection were 25 μg of LPS and 12.5 μg of proteins. Mice received a primary injection on day 0 and a secondary injection on day 14.
[c] Geometric means ± standard errors of PFC. Values are the results of three to four separate experiments.

TABLE 4. IgM and IgG subclass responses in mice to *E. coli* LPS alone and in combination with phospholipids, $M_r$ 39,000 protein, BSA, methylated BSA, or chymotrypsinogen[a]

| Immunogen[b] | Antibody type | PFC per $10^6$ spleen cells on day[c] | | IgG subclass (% of total IgG on day 19) | Adjuvant factor on day 19 |
|---|---|---|---|---|---|
| | | 18 | 19 | | |
| LPS | IgM | 45 ± 25 | 18 ± 5 | | |
| | IgG1 | 2 ± 1 | 3 ± 2 | 6 | 1 |
| | IgG2 | 4 ± 2 | 23 ± 4 | 43 | 1 |
| | IgG3 | 12 ± 4 | 27 ± 5 | 51 | 1 |
| LPS plus phospholipids | IgM | 44 ± 11 | 25 ± 11 | | |
| | IgG1 | 113 ± 27 | 175 ± 9 | 64 | 58 |
| | IgG2 | 27 ± 7 | 43 ± 11 | 16 | 2 |
| | IgG3 | 49 ± 10 | 56 ± 20 | 20 | 2 |
| LPS plus $M_r$ 39,000 protein | IgM | 51 ± 10 | 22 ± 6 | | |
| | IgG1 | 16 ± 3 | 23 ± 3 | 7 | 8 |
| | IgG2 | 129 ± 33 | 250 ± 20 | 81 | 11 |
| | IgG3 | 26 ± 17 | 35 ± 13 | 12 | 1 |
| LPS plus BSA | IgM | 24 ± 10 | 13 ± 1 | | |
| | IgG1 | 40 ± 31 | 51 ± 13 | 9 | 17 |
| | IgG2 | 371 ± 25 | 431 ± 12 | 71 | 19 |
| | IgG3 | 99 ± 57 | 122 ± 25 | 20 | 5 |
| LPS plus methylated BSA | IgM | 110 ± 10 | 49 ± 11 | | |
| | IgG1 | 184 ± 38 | 190 ± 10 | 55 | 63 |
| | IgG2 | 62 ± 29 | 64 ± 15 | 18 | 3 |
| | IgG3 | 49 ± 11 | 94 ± 9 | 27 | 3 |
| LPS plus chymotrypsinogen | IgM | 108 ± 11 | 40 ± 11 | | |
| | IgG1 | 370 ± 10 | 334 ± 42 | 74 | 111 |
| | IgG2 | 59 ± 39 | 40 ± 12 | 9 | 2 |
| | IgG3 | 60 ± 12 | 77 ± 4 | 17 | 3 |

[a] Responses were measured against *E. coli* LPS coupled to SRBC.
[b] Dosages per injection were 12.5 μg of LPS, 300 μg of phospholipids, and 12.5 μg of proteins. Mice received a primary injection on day 0 and a secondary injection on day 14.
[c] Geometric means ± standard errors of PFC. Values are the results of two to four separate experiments.

percentages of both the IgG2 and the IgG3 PFC were lower.

In the case of LPS mixed with the $M_r$ 39,000 protein, 62% IgG2 PFC were induced compared with 44% IgG2 PFC when LPS was injected alone. The LPS-lipoprotein mixture in turn induced predominantly IgG1 PFC specific for LPS.

The adjuvant factors for the effects of phospholipids and the outer membrane proteins on IgG subclass responses (Table 2, last column) were calculated by dividing the number of plaques belonging to the various IgG subclasses on day 19 obtained after immunization with LPS alone (taken as factor of 1) into the number of plaques belonging to the respective IgG subclasses obtained after immunization of mice with LPS-phospholipids or LPS-proteins. Phospholipids and the lipoprotein had the greatest effect on the stimulation of IgG1-antibody-producing cells to LPS, and the $M_r$ 39,000 protein had the greatest effect on IgG2-producing cells, although significant booster effects were observed in all three subclasses.

To determine whether nonbacterial proteins also induce alterations in the IgG subclass responses to LPS, mice were immunized with 25 μg of *P. mirabilis* D52 LPS I mixed with 12.5 μg of either BSA, methylated BSA, or chymotrypsinogen (Table 3). LPS-BSA preparations induced predominantly IgG2 PFC specific for LPS, while LPS-methylated BSA and LPS-chymotrypsinogen preparations induced mainly IgG1 PFC.

It should be noted that we could not detect IgG PFC directed against uncoupled SRBC or SRBC coupled with heterologous LPS at any time during the course of immunization with these LPS-phospholipid or LPS-protein preparations (data not shown). This confirms our previous findings using polyspecific rabbit anti-mouse immunoglobulin sera for development of IgG PFC (7, 21).

**Responses in mice to *E. coli* LPS preparations.**

It has been reported in the literature that immunization of mice with TNP-LPS from *E. coli* induced approximately equal numbers of IgG2 and IgG3 PFC and only small numbers of IgG1 PFC (23).

To determine whether the stimulation of predominantly IgG1 and IgG2 PFC was a peculiar feature of our *P. mirabilis* LPS I, we immunized mice with LPS isolated from a capsuleless, smooth strain of *E. coli* (20). For these experiments, different groups of mice were injected intraperitoneally with either LPS alone or LPS mixed with either phospholipids, $M_r$ 39,000 protein, BSA, methylated BSA, or chymotrypsinogen (Table 4). Phospholipids and the $M_r$ 39,000 protein were isolated from *P. mirabilis* 19. The amount of *E. coli* LPS injected (12.5 μg per injection) was chosen from preliminary dose-response experiments because it gave a minimal but still detectable response similar to our *P. mirabilis* LPS I. Considering the response of mice to *E. coli* LPS alone, the results were very similar to those obtained by Slack et al. (23); i.e., approximately equal numbers of IgG2 and IgG3 PFC and very small numbers of IgG1 PFC were induced. However, when adjuvants were mixed with this LPS before injection, alterations in the IgG subclasses were very similar to those observed with *P. mirabilis* LPS I as antigen. This demonstrates that the phenomenon is not restricted to a particular type of LPS.

Once again, the IgG responses were highly specific for *E. coli* LPS, as no IgG plaques were detected with uncoupled SRBC or SRBC coupled with heterologous LPS (data not shown).

**Responses in mice to *P. mirabilis* D52 LPS II preparations.** Our results with *P. mirabilis* D52 LPS I and *E. coli* LPS suggested that LPS structure may play a role in the induction of IgG-antibody-producing cells belonging to particular subclasses when LPS was administered alone. LPS I from *P. mirabilis* contains relatively short O-polysaccharide chains (one to two repeating units), while LPS II from this organism contains long O-polysaccharide chains, representing a more smooth form of LPS (3). The serological specificities of LPS I and LPS II from the same bacterium are identical in hemagglutination and in plaque test assays (H. Karch, unpublished data) so that with these two types of LPS, we had a means of testing the effect of the length of the O-polysaccharide chain on the IgG responses.

Table 5 presents the results of experiments measuring the responses to *P. mirabilis* D52 LPS II alone or LPS II mixed with either phospholipids or the $M_r$ 39,000 protein. The IgG subclass responses to LPS II alone were more similar to the responses to *E. coli* LPS than to those of LPS I; i.e., predominantly IgG3 and IgG2 PFC were induced. Once again, however, the tendency to stronger IgG1 production when LPS II was combined with phospholipids and to IgG2 production with LPS-$M_r$ 39,000 protein was observed.

**Effect of injecting LPS and adjuvants separately.** A series of experiments was performed to determine whether LPS and adjuvants had to be mixed and injected together to obtain the observed effects. Table 6 presents the results of sonicating *P. mirabilis* D52 LPS I and either phospholipids, $M_r$ 39,000 protein, BSA, or methylated BSA separately and injecting LPS and the adjuvants simultaneously but in different intraperitoneal sites (LPS in the left side, adjuvants in the right side). The amounts of LPS and adjuvants were the same as in the other experiments.

Essentially, the pronounced effects on the alteration of IgG subclass or augmentation of the responses obtained with mixtures of LPS and adjuvants were not observed in this experiment. Phospholipids injected separately did have a significant effect on the augmentation of IgG1 responses (adjuvant factor of 13), but this was quite small in comparison to the effect produced by mixtures of LPS and phospholipids before injection (adjuvant factor of 105, Table 2). In this regard it should be noted that the amount of phospholipids injected (300 μg) was large in comparison to the amount of proteins injected (12.5 μg) so that the possibility of mixing of LPS and phospholipids in the peritoneal cavity was no doubt greater and could account in part for the effects observed.

The results nevertheless indicate that mixing of LPS and adjuvants before injection was necessary to obtain pronounced effects of augmentation and alteration of particular IgG subclass responses to LPS.

## DISCUSSION

The characteristics of the IgG responses to LPS depended upon the type of LPS used and the nature of adjuvants used in complex with LPS as antigen. LPS containing short O-polysaccharide chains (one to two repeating units) induced approximately equal numbers of IgG1 and IgG2 PFC specific for LPS, while IgG3 cells were detected in much smaller numbers. In contrast, LPS containing long O-polysaccharide chains induced predominantly IgG2 and IgG3 PFC and comparatively small numbers of IgG1 cells. When adjuvants were complexed with LPS, predominantly IgG1 or IgG2 responses to both types of LPS were greatly augmented, depending upon the nature of the adjuvant.

The results suggest that the hydrophobic-hydrophilic character or perhaps the state of aggre-

TABLE 5. IgM and IgG subclass responses in mice to *P. mirabilis* D52 LPS II alone and in combination with phospholipids and $M_r$ 39,000 protein[a]

| Immunogen[b] | Antibody type | PFC per $10^6$ spleen cells on day[c] | | IgG subclass (% of total IgG on day 19) | Adjuvant factor on day 19 |
|---|---|---|---|---|---|
| | | 18 | 19 | | |
| LPS | IgM | 27 ± 1 | 14 ± 1 | | |
| | IgG1 | 6 ± 1 | 14 ± 4 | 19 | 1 |
| | IgG2 | 13 ± 4 | 25 ± 7 | 34 | 1 |
| | IgG3 | 13 ± 4 | 35 ± 3 | 47 | 1 |
| LPS plus phospholipids | IgM | 35 ± 2 | 21 ± 5 | | |
| | IgG1 | 112 ± 11 | 210 ± 60 | 60 | 15 |
| | IgG2 | 54 ± 5 | 71 ± 2 | 20 | 3 |
| | IgG3 | 48 ± 14 | 72 ± 7 | 20 | 2 |
| LPS plus $M_r$ 39,000 protein | IgM | 39 ± 5 | 23 ± 3 | | |
| | IgG1 | 140 ± 46 | 146 ± 4 | 26 | 10 |
| | IgG2 | 240 ± 17 | 293 ± 37 | 51 | 12 |
| | IgG3 | 75 ± 20 | 132 ± 13 | 23 | 4 |

[a] Responses were measured against *P. mirabilis* D52 LPS II coupled to SRBC.
[b] Dosages per injection were 25 μg of LPS, 300 μg of phospholipids, and 12.5 μg of $M_r$ 39,000 protein. Mice received a primary injection on day 0 and a secondary injection on day 14.
[c] Geometric means ± standard errors of PFC. Values are the results of three to four separate experiments.

gation of the adjuvant may play a role in the preferential augmentation of IgG1 or IgG2 responses to LPS. When LPS was mixed with phospholipids, lipoprotein, or methylated BSA, all of which have known hydrophobic properties, predominantly IgG1 PFC specific for LPS were induced. This was also the case for chymotrypsinogen, which was relatively insoluble (slightly turbid solutions even after sonication). When the more hydrophilic BSA was mixed with LPS, this combination induced predominantly IgG2 PFC. This also occurred with LPS-

TABLE 6. IgM and IgG subclass responses in mice to *P. mirabilis* D52 LPS I injected with either phospholipids, $M_r$ 39,000 protein, BSA, or methylated BSA simultaneously but separately in different peritoneal sites[a]

| Immunogen[b] | Antibody type | PFC per $10^6$ spleen cells on day[c] | | IgG subclass (% of total IgG on day 19) | Adjuvant factor on day 19 |
|---|---|---|---|---|---|
| | | 18 | 19 | | |
| LPS plus phospholipids | IgM | 40 ± 10 | 11 ± 2 | | |
| | IgG1 | 205 ± 32 | 215 ± 38 | 55 | 13 |
| | IgG2 | 120 ± 16 | 125 ± 3 | 32 | 7 |
| | IgG3 | 62 ± 8 | 48 ± 9 | 12 | 7 |
| LPS plus $M_r$ 39,000 protein | IgM | 31 ± 6 | 10 ± 1 | | |
| | IgG1 | 20 ± 6 | 46 ± 4 | 61 | 3 |
| | IgG2 | 9 ± 2 | 20 ± 2 | 27 | 1 |
| | IgG3 | 5 ± 5 | 9 ± 1 | 12 | 1 |
| LPS plus BSA | IgM | 21 ± 3 | 9 ± 2 | | |
| | IgG1 | 31 ± 4 | 16 ± 9 | 55 | 0.9 |
| | IgG2 | 17 ± 3 | 10 ± 7 | 34 | 0.5 |
| | IgG3 | 18 ± 5 | 3 ± 3 | 10 | 0.4 |
| LPS plus methylated BSA | IgM | 19 ± 2 | 12 ± 3 | | |
| | IgG1 | 12 ± 2 | 35 ± 5 | 64 | 2 |
| | IgG2 | 3 ± 1 | 17 ± 5 | 31 | 0.9 |
| | IgG3 | 3 ± 3 | 3 ± 3 | 5 | 0.4 |

[a] Responses were measured against *P. mirabilis* D52 LPS I coupled to SRBC.
[b] Dosages per injection were 25 μg of LPS, 300 μg of phospholipids, and 12.5 μg of proteins. LPS was injected into the left side of the peritoneum, and the other components were injected into the right side. Mice received primary injections on day 0 and secondary injections on day 14.
[c] Geometric means ± standard errors of PFC. Values are the results of three separate experiments.

$M_r$ 39,000 protein mixtures. Although the $M_r$ 39,000 protein is an outer membrane protein, it has strong hydrophilic properties. It forms hydrophilic pores in model membrane phospholipid vesicles, whereas lipoprotein does not (19), and it is easily solubilized in aqueous buffers after brief sonication.

Thus, such factors as type of LPS used and whether LPS is administered in pure form or in complex with other outer membrane components can greatly affect the responses to this antigen.

The selective augmentation of particular classes of IgG antibodies formed in response to LPS could be of practical advantage in the defense against infections with gram-negative bacteria. It has, for example, been shown that human IgG antibodies belonging to the IgG1 and IgG3 subclasses are particularly effective in promoting phagocytosis and killing of *E. coli* (9).

In addition, the model systems characterized in the present report can be of value for further investigations on the regulation of isotype induction and switching in antibody-producing cells. The modulation of the IgG responses to LPS in our study seems to be determined in large part by the physical character of the LPS or LPS-adjuvant preparations. Whether epitope density of the LPS preparations, which may be affected by the alteration in the state of aggregation, plays a role in isotype induction is a subject for further investigation. Both the state of aggregation and the epitope density of TNP-polyacrylamide beads have been shown to affect the ability of this type 2 thymus-independent antigen to induce responses in CBA/N mice (15, 16).

Recent reports have indicated that T cells are involved in the augmentation of $IgG2_a$ responses to TNP-Ficoll (17, 18) or IgG1 and IgG2 polyclonal responses to LPS (12, 13). Since IgG1 and IgG2 responses to LPS were greatly augmented by the adjuvant preparations used in the present report, the possible involvement of T cells in these LPS-specific responses is now under investigation in our laboratory.

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