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Isotype of Protective Anti-*Salmonella* Antibodies in Experimental Mouse Salmonellosis

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Mice and a rabbit were immunized with heat-killed *Salmonella typhimurium* bacteria or with an O-4,12 antigen-specific octasaccharide-protein conjugate. Immunoglobulin isotypes of the antisera were tested for their capacity to protect mice against experimental salmonellosis. Antibodies of immunoglobulin M + A isotypes were more protective than the immunoglobulin G antibodies in each of the two pools of mouse sera. The same protective pattern was also seen with a rabbit antiserum elicited by the artificial octasaccharide-protein conjugate, i.e., with antibodies with the exclusive specificity for the O-4,12 antigen determinants of *S. typhimurium*.

Cell-mediated immunity is of major importance for the survival of the host in experimental salmonellosis (6). However, humoral antibodies also contribute to protection, especially in the initial phase of the infection. Passive immunization experiments have demonstrated the effectiveness of antibodies on crude extracts of rough bacteria (5), as well as of antibodies with the exclusive specificity for the O-antigen determinants of the lipopolysaccharide (LPS) molecule (17, 18).

We have previously compared the efficiencies of different anti-*Salmonella* immunoglobulin (Ig) isotypes in providing protection from experimental mouse salmonellosis (14). In these studies, IgM antibodies were much more protective than antibodies of any other Ig class. This finding was surprising, and its mechanism was difficult to explain. The possibility that the superiority of IgM was due to different specificities of the respective isotypes was not formally excluded. Even when the protective antisera were obtained after immunization with smooth O-4,12 *Salmonella typhimurium* and the protection was assumed to be due to the anti-O antibodies, the specificity of the protective antibodies could have been other than the O antigen, and such antibodies could have been predominantly present in the IgM fraction.

The present study was initiated to exclude this possibility. Firstly, two new pools of mouse antisera were prepared by immunization with whole O-4,5,12 bacteria. Secondly, a rabbit antiserum elicited by a *Salmonella* O-4,12-specific octasaccharide-protein conjugate was tested. The IgG antibodies of all these sera were poor in protection when compared with the IgM + A antibodies. We concluded that the difference in the protective capacity of the serum factors studied is likely to be due to the C_H regions (containing the isotype determinants) rather than the V regions (specificity determinants) of the antibody molecules.

MATERIALS AND METHODS

Bacteria. Smooth, moderately mouse-virulent *S. typhimurium* LT2 SH2183 cells with the O-antigenic formula 4,5,12 were used for both immunization of the mice and for challenge in all experiments. When given intraperitoneally to the mouse strain used, the 50% lethal dose (LD_{50}) was 2×10^5, assayed as described before (19).

Mice. (CBA × C57B1/6)F_1 mice bred at the National Public Health Institute were used in all infection assays at the age of 6 to 8 weeks.

***Salmonella* O antigen-specific saccharide-protein conjugates.** The O-4,12-specific oligosaccharide-protein conjugates were prepared and characterized as described earlier by Svenson et al. (16).

Immunization. The A/J and the AL/N mice were immunized intraperitoneally twice 30 days apart with 10^8 heat-killed *S. typhimurium* SH2183 cells. Ten days after the second injection the mice were bled, and the sera were pooled into pool 1 and pool 2, respectively. For preparation of the rabbit serum, a New Zealand white rabbit was injected with the O-4,12-specific octasaccharide-diphtheria toxin conjugate into the popliteal lymph node as described earlier by Svenson et al. (18). Both the primary dose and the booster doses on days 21, 78, and 87 were 20 μg (dry weight) per rabbit.

Antibody assays. The rabbit antibodies were measured by enzyme immunoassay (EIA) (1) essentially as described earlier (14). The antigens used were the homologous O-4,5,12LPS (5 μg/ml), isolated by the hot phenol-water method (21); rough LPS (5 μg/ml), corresponding to the complete core structure (chemotype Ra) of *Salmonella*, isolated from *S. typhimurium* (strain *his-515*) (9) by the phenol-chloroform-petroleum-ether method (2); and an O-4,12-specific dodecasaccharide-bovine serum albumin (BSA) conjugate (16). The antigens were diluted in 0.1 M Tris-hydrochloride buffer (pH 8.5) to coat the wells of microtiter plates (Lindbro Division, Flow Laboratories, Hamden, Conn.) by overnight incubation at room temperature. The sera were titrated in half-log dilutions, and the binding of antibody was detected with peroxidase-conjugated anti-rabbit Igs (IgG + IgM + IgA) (Cappel Laboratories, Cochranville, Pa.). The titer was given as the reciprocal of the dilution giving the absorbance of 0.5 at 492 nm. The mouse antibodies were measured by solid-phase radioimmunoassay (RIA) according to the method of Karjalainen and Mäkelä (4) by using the same antigens, the same antigen concentrations, and the same buffer as described above.

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Antibodies in different fractions were compared on the basis of "antibody units" derived in a simple one-to-one correspondence from the titers: a serum with an EIA or RIA titer of 1 would have 1 antibody unit per ml.

Separation of rabbit Ig isotypes by the DEAE-protein A method. Four milliliters of a serum was divided in half. Two milliliters of serum was diluted with 6 ml of water at 4°C and added to 10 g of wet DEAE-cellulose equilibrated with 0.01 M phosphate buffer to pH 8.0 (12). After a 60-min incubation at 4°C, the DEAE-cellulose was removed, and the supernatant was called the IgG fraction. Another 2 ml of serum was added at 4°C to 6 ml of 0.1 M phosphate buffer (pH 8.0). Seven milliliters of wet protein A-Sepharose was added, and the mixture was incubated at 4°C with occasional stirring. The sorbent was removed by filtration, and the filtrate was called the IgM + A fraction.

Separation of mouse Ig isotypes. The method of Seppälä (15) was used. Of each of the serum pools, 1.6 ml was mixed with 0.75 ml of 1 M Tris buffer (pH 8.0), and the mixture was slowly passed through a protein A column at 4.5 ml (bed volume), followed by 30 ml of the pH 8.0 buffer. This buffer was followed by 24-ml volumes of buffers with pH values of 5.5, 5.0, 4.5, 4.0, and 3.0; the effluent was monitored by optical density. Fractions coming out at pH values 8, 6.5, 5, 4.5, and 3 were called IgM + IgA, IgG1, IgG2a, IgG3, and IgG2b, respectively.

Mouse protection assay. The protection assay was performed as described earlier (14). The mice received intraperitoneally 200 μl of 10-fold dilutions of the Ig fractions in 0.9% NaCl; control mice received saline only. Groups of six mice were used in all experiments. The mice were challenged 3 h later by an intraperitoneal injection of exponentially growing bacteria. The dose was known to be ca. 10 times the LD_{50}. Control groups of mice received half-log dilutions of the bacteria for checking the LD_{50} in each experiment. The numbers of dead mice were counted at day 10 (corresponding to the early phase of infection), and the amount of the Ig fraction needed to protect 50% of the mice was calculated.

RESULTS

To compare the protective capacities of the IgM + A and IgG fractions of the rabbit antiserum to the O antigen-specific 4,12 octasaccharide-diphteria toxin conjugate (16, 18), different dilutions of them were given to mice before the intraperitoneal *Salmonella* challenge. This allowed us to calculate the amount of each fraction needed to protect 50% of the mice. The results (Table 1) indicate that the IgM + A antibodies were more protective than the IgG antibodies; 2.5 μl of the IgM + A fraction was needed for 50% protection, but the IgG fraction protected none of the six mice that had received the highest dose of 40 μl and none of those receiving smaller doses. From this figure and the dilution curve of the IgM + A fraction, we extrapolate that at least 220 μl of the IgG antibodies would be needed to give 50% protection. The antibody titers of the fractions measured by EIA to the homologous O-4,5,12 LPS (Table 1) indicated that the fractions contained closely similar amounts of anti-O-4,12 antibodies; the titer of the IgM + A fraction was 70, and the corresponding titer of the IgG fraction was 20. Only the O-4,12 octasaccharide was shared by the immunogen and the challenge bacteria on one hand and by the immunogen and the solid-phase antigen on the other. When the amount of antibody units (an EIA titer of 1 corresponding to 1 EIA unit per ml of serum) needed to give 50% protection was calculated from the above values, the IgM + A fraction was ≥25 times more protective than the IgG fraction.

TABLE 1. Protective capacity and antibody titers in fractions of rabbit antiserum to octasaccharide-difteriatoxin conjugate

Isotype	Antibody titer[a] to O-4,5,12 LPS	50% Protective dose[b] (μl)	EIA units[c] needed to give 50% protection
IgM + A	70	2.5	0.175
IgG	20	>40 (≥220)[d]	≥4.4

[a] Reciprocal of the dilution giving the absorbance of 0.5 at 492 nm in EIA (1), with O-4,5,12 LPS as coating antigen. Peroxidase-conjugated anti-rabbit immunoglobulins (IgG + IgM + IgA) were used to detect the antibodies bound to the antigen.

[b] Dose required for 50% survival of the mice challenged with 10 × LD_{50} of the O-4,5,12 bacteria.

[c] Based on the antibody titer to O-4,5,12 LPS (titer of 1 = 1 EIA unit per ml).

[d] All mice died with the 40-μl dose; by extrapolation the 50% protective dose was estimated to be at least 220 μl.

In the second set of experiments, A/J and AL/N mice were immunized with heat-killed *S. typhimurium* SH2183, and the antisera were pooled into pool 1 and pool 2, respectively. The protective capacities of the Ig fractions were titrated as above. In these pools most of the protective capacity was also in the IgM + A fraction (Table 2), although some protection was obtained with IgG. Thus, the volume of the IgM + A fraction of pool 1 needed for 50% protection was 0.3 μl, whereas the corresponding volume of the IgG fraction was 2.3 μl. In the case of pool 2, 0.4 μl of IgM + A gave 50% protection, whereas 15 to 23 μl of the IgG fractions was required for the same effect.

The antibodies to several antigens, the O antigen-specific conjugate, the homologous O-4,5,12 LPS, and a rough LPS in the different fractions were determined by a solid-phase assay with a radiolabeled anti-mouse Ig serum. The results shown in Table 2 indicate that the two fractions of pool 1

TABLE 2. Protective capacity and antibody titers in fractions of mouse antisera immunized with heat-killed *S. typhimurium* O-4,5,12 bacteria

Serum and isotype	Ra LPS	Antibody titer[a] to: O-4,5,12 LPS	Antibody titer[a] to: O-4,12 DS-BSA	50% Protective dose[b] (μl)	RIA units[c] needed to give 50% protection
Pool 1					
IgM + A	900	900	1,000	0.3	0.27
IgG	1,000	800	600	2.3	1.8
Pool 2					
IgM + A	ND[d]	400	1,500	0.4	0.16
IgG1	ND	800	700	23	18.4
IgG2a	ND	200	200	15	3.0
IgG2b + G3	ND	20	20	23	0.46

[a] Measured by solid-phase RIA (4) by using Ra LPS, smooth O-4,5,12 LPS, or O antigen 4,12-specific dodecasaccharide-BSA (DS-BSA) conjugate as coating antigens. The control BSA antigen showed no antibody binding.

[b] Dose required for 50% survival of the mice challenged with 10 × LD_{50} of the O-4,5,12 bacteria.

[c] Based on the antibody titer to O-4,5,12 LPS (titer of 1 = 1 RIA unit per ml).

[d] ND, Not done.

contained similar quantities of antibodies binding to either the O-4,5,12 LPS of the immunizing strain or to the O-4,12 oligosaccharide-protein conjugate. Whereas the LPS antigen can bind antibodies directed towards the rough core as well as the O polysaccharide of the LPS molecule, the oligosaccharide-BSA conjugate measures anti-O antibodies only. Both fractions also contained antibodies to the rough LPS, and again the titers of the two fractions were equal. Most of these anti-rough antibodies could be removed by absorption of the antisera with rough heat-killed *S. typhimurium* (LPS of type Ra) bacteria, leaving the anti-O titer almost intact (data not shown). The titers of the O-4,12-specific antibodies in fractions IgM + A and IgG1 of pool 2 were also almost the same, irrespective of the antigen used in the assay. The titers in the IgG2a fraction were ca. 25% of those in the IgG1 fraction but were similar for both O-4,12 antigens, whereas the titers in the IgG2b + IgG3 fraction were very low.

When the passive protection data were compared with the antibody concentrations of the fractions on the basis of antibody units, it was clear that the IgM + A antibodies were more efficient in protection than the IgG antibodies (Table 2). The IgM + A antibodies of pool 1 were sevenfold more protective than the corresponding IgG antibodies, and the IgM + A antibodies of pool 2 were ca. 100-fold more protective than the IgG and ca. 20-fold more protective than the IgG2a antibodies. The antibody concentration of the IgG2b + IgG3 fraction in pool 2 (Table 2) was too low to estimate the possible protective capacity of IgG2b and IgG3—the small degree of protection could be due to contamination by IgM.

DISCUSSION

Reports on the Ig class distribution of serum antibodies to the O-antigenic LPS are controversial. LPS has been claimed to elicit primarily if not exclusively IgM antibodies (7, 11, 20). However, Metcalf and O'Brien (8) used an Ig class-specific RIA and concluded that the LPS antibodies elicited in mice immunized with whole acetone-killed *S. typhimurium* bacteria were mainly IgG. Karch et al. (3) immunized mice with LPS isolated from *Proteus mirabilis* as such or complexed to various proteins; the antibody responses obtained were similar to those reported by Metcalf and O'Brien.

Our results (Table 2) suggest that IgM and IgG are equally represented in polyclonal antisera elicited in mice by whole heat-killed bacteria. This was true both of anti-O antibodies and also antibodies to rough LPS (pool 1). In addition, the IgG1 and IgM titers in pool 2 to the O-antigenic part of the LPS and to smooth LPS were remarkably equal. The next common anti-O isotype in pool 2 was IgG2a, whereas very little of the anti-O was present in IgG2b or IgG3.

The sera in which the protective capacities against mouse salmonellosis of different Ig isotypes have been compared (15; this paper) now include sera of mice or rabbits immunized with whole O-4,5,12 bacteria, sera of mice immunized with envelopes of rough bacteria, and sera of rabbits immunized with an exclusively O-4,12-specific oligosaccharide-protein conjugate. In each case, most of the protective capacity has been associated with IgM antibodies, and these have been, on the basis of antibody activity (units) determined in solid-phase assays, 10 to 100 times more protective than IgG antibodies. A possible trivial explanation of these findings is that the solid-phase assays used were grossly underestimating the IgM antibodies or overestimating the IgG antibodies. This does not seem likely, since IgM antibodies are more efficient than IgG antibodies in binding to their antigen (10), suggesting that the solid-phase assays would tend to overestimate rather than underestimate IgM antibodies. It is formally possible, however, that the two anti-Ig antibodies used in the solid-phase assays strongly preferred IgG. The similar slopes seen in the dilution series of IgM and IgG fractions (data not shown) argue against this. Furthermore, if the apparent superiority in the protective capacity of IgM + A antibodies in the rabbit serum shown in Table 1 were to be explained by such a bias, it would mean that less than 1% of anti-O-specific antibodies in the rabbit serum were of IgG. This is unlikely since other small carbohydrates coupled to protein carriers have been shown to induce predominantly IgG antibodies in the secondary response (unpublished data).

In all the three sera or serum pools (Tables 1 and 2), the bulk of the protective capacity was found in the IgM + A-containing fractions, whereas the antibody concentrations in these fractions were very similar to the antibody concentrations of the IgG fractions combined. Antibody concentrations among the fractions representing the different IgG subclasses, however, varied; in particular, the IgG2b or IgG3 titers were very low. Therefore, these data leave the protective capacity of the IgG2b and IgG3 isotypes open. However, it has been reported that an anti-O-specific hybridoma antibody of the IgG2b isotype was not protective in a similar infection model (A. D. O'Brien, W. E. Biddison, and E. S. Metcalf, Fed. Proc., p. 830, 1982).

The consistent demonstration by these and previous (14) experiments of the superior protective capacity of IgM antibodies has led us to inquire into its mechanism. These studies (H. Saxén, submitted for publication) suggest that the efficiency of IgM is due to the efficient activation of complement by the IgM bound to the bacteria, promoting rapid killing of the bacteria in the peritoneal cavity, presumably by the resident macrophages. Rowley and Turner (13) have, indeed, shown the higher efficacy of IgM antibodies in accelerating the intraperitoneal killing of *Salmonellae* sp., but the protective capacities of the isotypes were not compared.

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