Mechanism of the Protective Action of Anti-*Salmonella* IgM in Experimental Mouse Salmonellosis

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The kinetics of mouse salmonellosis caused by *Salmonella typhimurium* was studied in mice preinjected with the IgM or IgG fraction prepared from a rabbit anti-*Salmonella* serum. Compared on the basis of antibody units determined by an enzyme immunoassay, IgM was ten times more effective than IgG in promoting removal of the bacteria from blood after intravenous (IV) injection and their uptake in the reticuloendothelial system (RES). The subsequent killing of the bacteria was, however, only minor, in accord with the negligible protective effect of serum antibodies in IV infection. IgM was over 1000 times more effective than IgG in promoting killing of the bacteria after intraperitoneal (IP) challenge. Neither antibody had an effect on the multiplication of the bacteria in the RES. The protective action of antibody was thus almost entirely mediated by peritoneal-cavity cells acting in the very early phase of infection. The greater effect of IgM is suggested to be a special feature of *Salmonella* infections, connected with the capacity of these bacteria for intracellular survival and multiplication in the RES.

INTRODUCTION

Mouse salmonellosis has been extensively studied as a model of an infection caused by facultatively intraphagocytic bacteria (Ørskov *et al.*, 1928; Jenkin & Rowley, 1963; Mackaness *et al.*, 1966; Collins *et al.*, 1966), but many questions still remain open. Thus, genetic differences in both the host (Webster, 1937; Biozzi, 1972; Plant & Glynn, 1976; O'Brien *et al.*, 1979; Hormaeche *et al.*, 1980) and the pathogen (Ørskov & Moltke, 1928; Roantree, 1967; Valtonen, 1970; Hoiseth & Stocker, 1981) influence the outcome of the infection, but their exact mechanism of action is known only in the specific instance of genes causing a nutritional defect in the bacteria. Both cellular and humoral immunity play a role in the defence but their relative contributions are still under debate (Levine & Hornick, 1981). The route of infection influences the course of the infection, but we do not know, for example, why intraperitoneally (IP) induced infection is more easily prevented by antibodies than is infection after intravenous (IV) challenge (Mackaness *et al.*, 1966; Jenkin & Rowley, 1963; Blanden *et al.*, 1966).

Recent studies in this laboratory have revealed a further unexplained feature of experimental mouse salmonellosis: the antibodies protective against IP challenge were primarily of the IgM class (Saxén & Mäkelä, 1982; Saxén *et al.*, 1984*b*). This was true of antibodies of rabbit or mouse origin, induced by immunization with whole smooth bacteria, with cell envelopes of a rough mutant or with a semi-synthetic antigen in which an O-antigen-specific oligosaccharide was conjugated to an unrelated protein, diphtheria toxoid. Many of these sera contained approximately equal amounts of IgM and IgG anti-*Salmonella* antibodies, but some had much more IgG than IgM.

Why IgM has this special position in *Salmonella* infections is not clear. I have now approached this question by comparing the effects of anti-O-antibodies of the IgM and IgG class on the kinetics of the infection.

Abbreviations: IP, intraperitoneal(ly); IV, intravenous(ly); LD₅₀, 50% lethal dose; RES, reticuloendothelial system.

METHODS

Bacteria. The smooth, moderately mouse-virulent *Salmonella typhimurium* LT2 strain SH2183 with the O-antigenic formula 4,5,12 grown to late exponential phase (2×10^9 bacteria ml^{-1}) at 37 °C under aeration in L-broth (Miller, 1972) was used in all experiments. Its 50% lethal dose (LD_{50}) when given IP to the mouse strain used was 2×10^5 , assayed as described by Valtonen (1970).

Mice. (CBA $\times$ C57Bl/6) F_1 mice (6 to 8 weeks old) bred at this institute were used in all assays.

Antibody fractions. The hyperimmune rabbit serum K2249 and its fractions were derived as described by Saxén & Mäkelä (1982). The immunogen was heat-killed whole smooth O-4,5,12 *S. typhimurium* (approximately 10^8 bacteria per injection) injected in the footpads seven times over a period of 8 weeks. The IgG and the IgM plus IgA fractions were prepared from separate samples of serum. The IgG fraction was obtained by removing IgM and IgA by adsorption to DEAE-cellulose in 0.01 M-phosphate buffer, pH 8.0, and the IgM plus IgA fraction by adsorption of IgG to protein A-Sepharose. The contribution of IgA to the protective action of this serum was negligible (Saxén & Mäkelä, 1982), and thus the IgM plus IgA fraction was used as representative of IgM.

Enzyme immunoassay. The assay was performed exactly as described before using O-4,5,12 LPS antigen (Saxén & Mäkelä, 1982). The serum fractions were titrated in half-log dilutions and binding was detected with an alkaline phosphatase conjugated anti-rabbit-immunoglobulin (Orion Diagnostica, Espoo, Finland). The titre was taken as the dilution that gave an A_{400} of 0.4, and its reciprocal used to define the antibody concentration in enzyme immunoassay units (100 units ml^{-1}) corresponding to a titre of 1 in 100.

Blood clearance assay (Krishnapillai & Karthigasu, 1969). The mice received half-log dilutions of the antibody fractions (or saline) via the lateral tail-vein in a volume of 200 μl , followed 1 h later by radiolabelled bacteria given by the same route. The bacteria were iodinated (Na^{125}I , Amersham) just before use by the 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodo-Gen, Pierce Chemical Co., Rockford, Ill., USA) method (Fraker & Speck, 1978). The injected dose contained 10^8 bacteria (2×10^5 c.p.m.) in 200 μl . At various times after this, blood samples (50 μl each) were taken from the retro-orbital plexus under ether anaesthesia.

The mice were sacrificed by cervical dislocation at given times up to 15 min post challenge, and the livers and spleens removed. The specific activity of all the samples was determined in a gamma counter. For the calculations, the blood volume of a mouse was taken as 2 ml. Each point in the figures represents the geometric mean of the results from three mice.

Enumeration of viable bacteria in the tissues. Tenfold dilutions of the antibody fractions or saline were injected (IP) into mice in a volume of 200 μl , followed 3 h later with the challenge (500 μl usually containing 2×10^6 bacteria per mouse) corresponding to $10 \times \text{LD}_{50}$. In some experiments, both the antibody and challenge injections were given IV, each in a volume of 200 μl . The mice were kept in groups of six, and killed for sampling at the times indicated. Blood samples were taken as in the clearance assay. The fluid in the peritoneal cavity was collected by careful rinsing with 2 ml PBS (Jenkin & Rowley, 1965). The liver and spleen were cut out, put in plastic bags with 10 ml sterile PBS and homogenized for 2 min in a Colworth Stomacher 80 (Seward, London, UK). Tenfold dilutions of all the samples were subsequently prepared in water for viable counts and the numbers of viable bacteria per organ were calculated. Each point in the figures represents the geometric mean of the results from three mice.

Mouse protection assay. Groups of six mice were challenged with 200 μl of tenfold dilutions of the bacterial culture given IP or IV, as indicated, and the numbers of deaths were recorded during the following 10 d. To assay protection, parallel groups of mice were injected IP with 200 μl PBS, or the antiserum K2249 diluted 1 in 30, 3 h before or 24 h or 48 h after challenge, as indicated.

RESULTS

Effect of the antibodies on IV injected salmonellae

The opsonization activity of the rabbit IgG and IgM antibody fractions, both of which gave enzyme immunoassay titres 1 : 700 to homologous LPS, corresponding to 700 units of antibody ml^{-1} (Saxén & Mäkelä, 1982), was compared in an *in vivo* opsonization assay. Without antibody, the radiolabelled bacteria were slowly cleared from the blood; 50% of the label was still present in the blood 15 min after injection, whereas in the presence of either antibody fraction, most of the label had disappeared from the blood within 5 min (Fig. 1a). At the same time, a large part of the label appeared in the livers of antibody-treated mice (Fig. 1b). When the effect of the two Ig fractions was assessed 5 min after challenge, IgM was tenfold more potent than IgG in accelerating the blood clearance and the liver uptake of the radiolabelled bacteria (Fig. 2).

Two hours after IV injection of 4×10^5 bacteria, none were found in the blood. The numbers of viable bacteria in the liver and spleen (Fig. 3) indicated very little, if any, immediate killing.

Fig. 1. Effect of anti-*Salmonella* IgG or IgM antibodies on the blood clearance of radiolabelled *S. typhimurium* shown as the fraction of label remaining in the blood or taken up by the liver at 1–15 min after IV injection. The mice received saline (□) or 20 antibody units of the IgG (○) or the IgM (●) fraction of the anti-*S. typhimurium* antiserum IV 1 h before challenge. Each point represents the geometric mean of results for three mice; the vertical line shows the scatter between the values.

Fig. 2. A quantitative comparison between anti-*Salmonella* IgG (—) and IgM (---) antibodies needed to promote removal of the bacteria from the blood or their uptake into the liver 5 min after IV injection of radiolabelled *S. typhimurium*. The experimental procedure was as described in Fig. 1, except that the mice received graded doses of the antibody fractions IV 1 h before challenge.

After the first 5 h a steady state was reached and then the bacterial numbers increased at a slow but even rate over the following days. The apparent rate of growth in the RES was not affected by varying the challenge dose between 10^5 and 10^7 (data not shown). Preinjection of either IgG or IgM increased the initial killing to a minor extent only, and had no effect on subsequent growth.

Effect of antibodies on the kinetics of mouse salmonellosis after IP challenge

Approximately 2×10^6 bacteria were injected IP, and their subsequent fate monitored by viable counts. There was little killing (or removal) of the bacteria in mice which had not received antibodies, and 60% of the inoculum could be recovered 1 h after injection. A large dose (20 antibody units) of either IgG or IgM reduced the number of viable bacteria to approximately 1% of the inoculum. Titration of the antibody fractions showed that this effect was obtained with 2 units of IgG and <0.002 units of IgM. The latter was thus a 1000-fold more efficient opsonin for intraperitoneal killing – a much larger difference than seen when testing opsonization by the blood clearance assay (Fig. 2). The antibody effect in the peritoneum was restricted to the first hour: over the following 23 h the bacterial numbers increased slowly, the rates being similar for IgG- and IgM-treated mice.

The initial stages of bacterial appearance in the liver and spleen could not be followed accurately because of contamination of the surfaces of these organs by the peritoneal contents.

Table 1. *Antibody-mediated protection from IP *S. typhimurium* infection*

Animal treatment (IP)		No. of mouse deaths† at a challenge dose of LD ₅₀ × :		
		100	10	1
Saline	3 h before challenge	6	5	4
Antiserum*	3 h before challenge	3	0	0
	24 h after challenge	6	6	6
	48 h after challenge	6	6	6

* 200 µl serum K2249 diluted 1:30, containing 20 antibody units of both IgG and IgM.

† No. of mice dead 10 d after challenge with graded dose of strain SH2183: six mice per group.

However, 1 d after IP injection there were as many bacteria in these organs as in the peritoneal cavity. When the efficacy of the antibody fractions in reducing the numbers of bacteria in the liver 1 d after an IP injection was titrated, the result closely paralleled that seen for IP killing. A 100-fold reduction was effected by the IgM fraction tested at concentrations of 0.06, 0.6, 6 and 60 antibody units. By contrast, only the largest dose, 60 units, of the IgG fraction produced the same effect, whereas doses of 0.06, 0.6 and 6 units gave liver counts indistinguishable from those seen after saline only. The same, maximally 100-fold reduction was seen with challenge doses varying from 6×10^4 to 2×10^6 .

On the subsequent days (1 to 4) the bacterial population in the liver and spleen increased slowly in the same manner as seen after IV challenge. This apparent growth was affected little if at all by preinjection of antibodies for each of the doses of IgG or IgM between 0.06 and 60 units.

Thus, the effect of antibodies on IP infection was restricted to the first day after challenge. To test whether this restriction also applied to the protective effect of the antibodies, groups of mice were challenged with IP injection of 10 and $100 \times \text{LD}_{50}$ doses of the bacteria and injected with a high dose of antiserum (> 20 antibody units of both IgG and IgM per mouse) either 3 h before or 24 or 48 h after the challenge. There was excellent protection from death by the preinjection of antiserum, but none by the later injections (Table 1).

DISCUSSION

The effect of antibodies in mouse salmonellosis is restricted in several ways: (i) it is clearcut against IP but negligible against IV challenge (Mackaness *et al.*, 1966; Jenkin & Rowley, 1963; Blanden *et al.*, 1966); (ii) it is almost exclusively mediated by the IgM class of anti-*Salmonella* antibodies (Saxén & Mäkelä, 1982; Saxén *et al.*, 1984b); and (iii) as shown in the present study, their effect is restricted to the very early phase of the infection. The data presented here help to explain each of these restrictions by showing where in the infectious process the antibodies intervene.

Both the IgM and IgG anti-*Salmonella* antibodies were active opsonins in the *in vivo* blood clearance assay. The IgM-promoted uptake of the bacteria must have taken place via the C3b receptor of the phagocytes, since macrophages, which are believed to be the main effector cells in *Salmonella* defence (Akeda *et al.*, 1981; O'Brien *et al.*, 1979b) do not have receptors for IgM (Bianco *et al.*, 1975). Indeed, uptake of *Salmonella* by mouse macrophages is promoted by the activation of complement *via* the alternative pathway without the need of antibodies (Liang-Takasaki *et al.*, 1982; 1983a). The slow clearance of the bacteria observed in the non-antibody-treated mice (50% in the first 15 min) was probably also mediated by C3b deposition as a result of a slow activation of the alternative pathway of complement by the LPS of the *S. typhimurium* (Liang-Takasaki *et al.*, 1983b).

IgM was, as previously known (Robbins *et al.*, 1965), an approximately 10-fold more active opsonin than IgG (Fig. 2), suggesting that it was more effective in activating complement, as shown in other particle-bound systems (Frank *et al.*, 1975; Borsos & Rapp, 1965). However, even if the antibodies promoted uptake of the opsonized bacteria in the RES, subsequent killing of the bacteria was inefficient and a steady multiplication of the bacteria started very soon (Fig. 3).

Fig. 3. Effect of 20 antibody units of anti-Salmonella IgG (○) or IgM (●) antibodies on the number of live bacteria in the liver or spleen of mice after IV injection of 4×10^5 *S. typhimurium*. The control mice received saline (□). The challenge dose is indicated by an arrow. Each point represents the geometric mean of results for three mice.

Table 2. Comparison of the efficacy of the IgM and IgG fractions of anti-Salmonella serum in different effector functions

Effector function	Challenge route	No. of units required	
		IgG	IgM
50% Clearance within 5 min (Fig. 3)	IV	12	1.2
>90% Reduction of bacteria in peritoneal fluid	IP	2	<0.002
99% Reduction of bacteria in liver	IP	60	≤0.06
50% Protection after challenge with $10 \times \text{LD}_{50}$ *	IP	11	0.015

* See Saxén & Mäkelä (1982).

When the bacteria were injected IP into non-immune mice, the numbers of bacteria recovered 1 h after challenge were close to those of the inoculum, indicating that unopsonized bacteria were not removed or killed in the peritoneum. Preinjection of antibodies caused a rapid decline in the numbers of viable bacteria in the peritoneal cavity, and IgM was >1000-fold more efficient than IgG. These data are in accord with previous findings (Rowley & Turner, 1966; Briles *et al.*, 1981), which also showed, by comparing viable bacterial counts with radioactive label, that the opsonized bacteria were killed and not just lost from the sample. As discussed above, the IgM could only act via complement activation, and these results suggest a primary role for C3b in killing of opsonized salmonellae in the peritoneal cavity.

The antibody-dependent early reduction in bacterial numbers in the peritoneal cavity could in fact explain the whole protective effect of the antibodies. It led to a reduced initial number of bacteria in the liver and spleen, the main sites of bacterial multiplication in mouse salmonellosis (Collins, 1969). Since the intracellular growth rate was not affected, this resulted in a reduced final number of bacteria and protection from death. Indeed, both the qualitative and quantitative antibody requirements for the intraperitoneal killing of the bacteria and the protective effect were very similar (Table 2).

Considering the often expressed doubts about a protective effect of antibodies in mouse salmonellosis, the very small amounts of IgM that were active against IP challenge (Table 2) are remarkable. The protective dose of 0.015 antibody units corresponds to 1 ml of a 1 in 200 000 dilution of the rabbit immune serum. Rowley & Turner (1966) showed that a corresponding rabbit anti-Salmonella serum had 66 µg protein in the IgM fraction from 1 ml anti-O serum, and promoted killing of IP injected salmonellae at a dilution of 1 in 500 000. From these figures they calculated that eight molecules of IgM (but 2000 molecules of IgG) per bacterium were sufficient for opsonization.

A comparison of the infection kinetics after IV and IP challenge showed that in both cases the main site of bacterial multiplication was the RES where the apparent growth rate of the bacteria between 1 and 4 d after infection was the same. The only difference was in the early handling of the bacteria: the macrophages in the liver and spleen were inefficient at killing the bacteria they ingested (and the killing was not improved by opsonization) whereas in the peritoneum the bacteria were efficiently killed. The latter were, however, dependent on opsonization via IgM and complement. The effector cells in the peritoneum capable of causing (in co-operation with complement) the observed killing of salmonellae were, however, not identified and experiments to elucidate this question are in progress.

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