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Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for *Salmonella enteritidis* and *S. typhimurium*

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The efficacy of chicken egg yolk homotypic antibodies specific for outer membrane proteins (OMP), lipopolysaccharide (LPS) or flagella (Fla) in controlling experimental salmonellosis in mice was investigated. Mice challenged orally with 2×10^9 *c.f.u. of* Salmonella enteritidis *or* 2×10^7 *c.f.u. of* S. typhimurium *were orally treated with 0.2 ml anti-OMP, -LPS or -Fla yolk antibody three times a day for three consecutive days. In mice challenged with* S. enteritidis, *antibody treatment resulted in a survival rate of 80%, 47% and 60% using OMP, LPS or Fla specific antibodies respectively, in contrast to only 20% in control mice. In the* S. typhimurium *trial, survival rate was 40%, 30% and 20% using OMP, LPS or Fla specific antibodies respectively in contrast to 0% in control mice.* In vitro *adhesion of* S. enteritidis *and* S. typhimurium *to HeLa cells was significantly reduced by anti-OMP, -LPS, and -Fla homotypic antibodies. Results suggest that egg yolk antibodies specific for Salmonella OMP, LPS, and Fla may protect mice from experimental salmonellosis when passively administered orally. Of these antibodies, anti-OMP exhibited the highest level of protection* in vivo *and* in vitro. © *1998 Elsevier Science Ltd. All rights reserved*

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Salmonella enteritidis and *S. typhimurium* are a non-host restricted serotype which can cause disease syndromes like gastroenteritis and systemic infections in a wide range of animal species. In the distal ileum, salmonellae adhere to and pass through intestinal epithelial cells, primarily the M cells of the follicle-associated epithelium[1–3].

Many approaches have been employed to investigate the mechanisms of pathogenicity of *Salmonella*[4–8]. Genetic and molecular studies on plasmid and genes encoding for virulence determinants have been reported[9–11]. *In vitro* studies of bacterial adhesion and invasion have been facilitated by tissue culture assays on isolated intestinal epithelial cells and epithelial cell monolayers[12–16].

Salmonella has a variety of surface components which are virulence-related. Among them, OMP also known as porins, play a role as pathogenicity determinants[17]. OMP are exposed on the surface of the bacterial cell and they can serve as phage receptors and react with antibodies[18]. Numerous studies have elucidated their biological functions and immunogenic properties[19,20]. With their physical and biological characteristics, OMP have been used successfully as vaccine antigens and a number of workers have proven that they are good immunogens and are protective in both active and passive immunization studies involving mice with sera as the source of protective antibodies[18,21–24]. OMP and LPS are required to elicit an effective cellular immune response and antibodies with immunoprotective potential[25,26]. Monoclonal antibodies that presumably recognized epitopes present in porin-LPS complexes were also protective against endotoxemia and mouse typhoid[27].

Motility seems to increase the probability that bacteria will reach suitable sites for invasion[28,29]. Active flagella facilitate movement towards host cells *in vitro*. The absence of flagella during infection does not reduce the ability of salmonellae to enter cultured cells or initiate disease by penetration of the Peyer's patches, and the presence of inactive flagella seems to

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attenuate the ability of the organism to interact with the host[30].

A correlation between the presence of fimbriae and bacterial virulence has been demonstrated in salmonellosis with a range of fimbrial types[31]. Conversely, decreased virulence was due either to loss of fimbriation or to the inhibitory action of anti-fimbrial antibodies on bacterial adhesion[32]. In another study, bacterial virulence was reduced by passive immunization with chicken egg yolk anti-fimbrial antibodies[33].

The aim of the present study was to determine the efficacy of chicken egg yolk derived antibodies specific for salmonellae OMP, LPS, and Fla in the control of experimental salmonellosis in mice and in adhesion inhibition *in vitro*. In the *in vivo* trial, the effect of passive immunization of anti- OMP, -LPS, and -Fla antibodies on the excretion pattern and persistence of *S. enteritidis* and *S. typhimurium* in infected mice was characterized.

MATERIALS AND METHODS

Mice

A total of 100 BALB/c SPF mice (Charles River Japan Inc., Kanagawa, Japan) 4 weeks old were randomly distributed into two trials.

Bacterial strains

S. enteritidis strain GIFU-3161 (ATCC-13076) and *S. typhimurium* strain GIFU-4892 (ATCC-13311) were obtained from Dr T. Ezaki, Gifu University, School of Medicine, Gifu City, Japan. These isolates were grown in Luria broth at 37°C for 18 h. For inactivation, whole bacteria were suspended in 0.5% formol for 18 h at 37°C in PBS.

Preparation of outer membrane proteins

OMP was prepared using the method of Nurminen[34] and Schnaitman[35] with modifications. Bacteria were suspended in 0.01 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer and homogenized. After centrifugation at 12000 *g* at 4°C, the bacterial cells were resuspended in 0.01 M HEPES and sonicated. Centrifugation was repeated and supernatant was collected and subjected to ultracentrifugation at 200000 *g* for 45 min at 4°C. Bacterial pellet was treated with 2% triton-X100 in 0.01 M HEPES and ultracentrifuged. The procedure was repeated with 2% triton-X100 and 5 mM EDTA in 0.01 M HEPES, and ultracentrifuged. The sediment was resuspended in PBS and dialysed against the same buffer. The protein content of the sample was determined by a Bio-Rad protein assay system (Bio-Rad Laboratories, Calif., USA). The purity of each OMP preparation was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5–20% gradient acrylamide gels (Atto Co., Tokyo, Japan) with prestained standards (Bio-Rad).

Preparation of lipopolysaccharide

The conventional phenol extraction procedure was used[36]. Briefly, bacteria cells were suspended in distilled water. An equal volume of 90% (W/V) phenol preheated to 68°C was added, and the resultant mixture was stirred for 20 min and centrifuged. Aqueous phases were centrifuged, dialysed against distilled water, centrifugated again and lyophilized.

Crude LPS preparations were suspended in 10 mM Tris-HCl buffer (pH 7.5) with NaCl concentrations 10 mM. The LPS was applied onto a ion-exchange chromatographic column packed with Q Sepharose First flow (Pharmacia Biotech, Uppsala, Sweden). For fractionation, 10 mM Tris-HCl buffer with increasing NaCl concentrations (0.2, 0.3, 0.4, and 1.0 M) was used as eluent. Fractions were collected and protein was monitored spectrophotometrically at 280 nm. The solution was dialysed against PBS and the LPS preparation was analysed by SDS-PAGE.

Preparation of flagella

The conventional homogenized extraction procedure was used. Bacteria were suspended in PBS and homogenized for 2 min. After centrifugation to remove the cells, the supernatant was collected and subjected to ultracentrifugation at 40000 *g* for 4 h. The pellet was treated in 0.1 M Tris-HCl buffer (pH 8.0). The flagella was applied onto an ion-exchange chromatographic column packed with DEAE-Sephadex A-50 (Pharmacia). For fractionation, 0.1 M Tris-HCl buffer (pH 8.0) with increasing NaCl concentrations (0.3, 0.5, 0.7, and 1.0 M) was used as eluent. Fractions were collected and protein was monitored spectrophotometrically at 280 nm. The solution was dialysed against PBS and the protein content and purity of the pooled sample was determined. Purity of the flagellar preparation was analysed by SDS-PAGE and further confirmed by transmission electron microscope (H-300, Hitachi, Tokyo, Japan) examination of negatively stained samples.

Immunization of chickens

Five-month-old White leghorn chickens (strain Hyline W36; GHEN Corporation, Gifu, Japan) were immunized with OMP, LPS, and Fla extracted from *S. enteritidis* and *S. typhimurium* for the induction OMP, LPS, and Fla specific antibodies in egg yolk. The antigen consisting of 0.5 mg of either OMP, LPS or Fla was emulsified in emulsion oil mixed with 5% sorbitan monooleate (Maine Biological Laboratories, Maine, USA) as adjuvant and injected intramuscularly into the breast muscles of chickens. Eight weeks after the first injection, chickens received booster injections of the same dosage by the same route of administration and this was repeated two weeks later. Eggs were collected daily starting from the second week after the last booster until enough eggs were pooled and processed.

Isolation of antibodies from chicken egg yolk

The egg yolk Immunoglobulin G (IgG) was prepared using the procedure in an earlier study[37]. The separated yolks were diluted with 8 volumes of distilled water. An aqueous suspension of 5% hydroxypropylmethylcellulose phthalate (Shinetsu Chemical Inc., Tokyo, Japan) in 80% ethyl alcohol was added to the diluted egg yolk gradually, and the mixture was centrifuged at 12000 *g* for 20 min. The supernatant which contained the IgG fraction was purified by affinity chromatography using

Avid AL gel (BioProbe International Inc., Tustin, CA, USA). Elution of the sample was accomplished by 0.05 M sodium acetate buffer (pH 3.0). The solution was dialysed against PBS, and analysed for antibody titer and purity by SDS-PAGE.

Titration of antibodies

The antibody titer against the challenge-exposure strain was determined by a microtitration plate agglutination method. Inactivated whole bacteria of *S. enteritidis* and *S. typhimurium* were used as antigen in the agglutination test. Samples of bacterial suspension were added to PBS to a desired optical density (0.42 at 620 nm). The adjusted bacterial suspension was used as antigen; 0.05 ml was added to equal volumes of twofold serial dilutions of an antibody solution in PBS. After 2 h incubation at 37°C and overnight incubation at 4°C, the titer was read as the highest dilution of antibody showing agglutination.

Challenge exposure of mice to *Salmonella* and oral administration of antibodies

The succeeding procedures in the challenge exposure of mice were performed as described elsewhere[33]. Trial 1: Four groups with 15 mice per group was inoculated orally with 0.2 ml of *S. enteritidis* suspension (1×10^{10} c.f.u. ml^{-1}). Trial 2: Four groups with ten mice per group were inoculated orally with 0.2 ml of *S. typhimurium* (1×10^{8} c.f.u. ml^{-1}). All mice in each trial were given 0.2 ml of the challenge infectious dose as specified above and about 30 min later, 0.2 ml antibody solution consisting of anti-OMP, anti-LPS, anti-Fla and control antibody from sham immunized chickens were given to groups 1, 2, 3, and 4 respectively. The antibody solution was given daily to each mouse three times a day for three consecutive days. The bacterial inoculum and antibodies were administered by gastric intubation using a sterilized blunt needle attached to a 1 ml tuberculin syringe. Mice were observed daily for two weeks for clinical signs of infection and survival (%) of mice was calculated.

Enumeration of *Salmonella* in organ homogenates

At the end of the trial, surviving mice were killed by quick cervical dislocation and spleen, liver, and cecum were collected. The organ samples were homogenized in PBS, subcultured in protein water (Difco, Detroit, Mich., USA), enriched in Selenite broth (Nissui Pharmaceutical Co., Tokyo, Japan) incubated, and then cultured on desoxycholate-hydrogen sulfide-lactose agar (DHL agar: Nissui) and confirmed by positive O4 and O9 antiserum agglutination test (Denka Seiken Co., Tokyo, Japan).

In vitro adherence of *Salmonella* to HeLa cell and inhibition by antibodies

The succeeding procedures in the adhesion assay were performed as described elsewhere[16] with modification. Bacteria were suspended at a concentration of approximately 1×10^{7} c.f.u. ml^{-1} in Eagle's minimal essential medium without kanamycin (MEM No.3, Nissui). HeLa S3 cell monolayers (ATCC CCL-2.2; Flow Laboratories, McLean, Va., USA) were prepared by placing 10^{5} cells in MEM medium into each chamber of Lab-TeK tissue culture chamber/slides (Lab-Tek, #4804, Miles Lab., Naperville, Ill., USA). After a period of incubation, the medium was aspirated from each chamber and 1 ml suspension of *Salmonella* or *Salmonella*+antibody was added. Inhibition of bacterial attachment to HeLa cells was done by pre-incubating the mixture of 1 ml of bacteria with 1 ml of egg yolk antibody solution in MEM (1:10 dilution of original purified IgG sample) for 15 min and followed by twofold serial dilution of the mixture. One millilitre of the serially diluted mixture was added onto Lab-Tek chambers with HeLa cell as described above followed by incubation at 37°C for 30 min. The slide chambers were then washed thrice with warm PBS, fixed with cold acetone, and stained with Giemsa solution. The number of bacteria attached to 20 HeLa cells was determined and the average number of bacterial cells attached to each HeLa cell was calculated. The minimum adhesion inhibitory concentration titer was determined by the formula: antibody titer/dilution of antibody solution with significant ($p < 0.01$) adhesion-inhibition assay.

Statistical analysis

The statistical significance of differences in survival rates between the treated and control groups was assessed by using the Fisher's exact test, and the mean differences in bacterial adhesion *in vitro* between the control and pre-incubated groups was assessed by the Student's *t* test.

RESULTS

Antibody titer to *salmonella*

The immunological specificity of egg yolk antibody was assessed by a microtitration plate agglutination method. The titer of the OMP, LPS, and Fla specific antibody solution for *S. enteritidis* were 256, 128, and 1024 respectively (*Table 1*). Those for the OMP, LPS, and Fla antibody solution for *S. typhimurium* were 256, 128, and 2,048 respectively (*Table 2*). The titer of egg yolks of non-immunized hens was <2 against the inactivated whole bacteria antigens used in the assay.

Clinical evaluation of mice after challenge exposure with *Salmonella* and passive immunization with antibody

Trial 1. The clinical responses of mice after *S. enteritidis* challenge and subsequent treatment with antibodies are shown in *Figure 1*. Mice in Group 1 (OMP antibody: titre 1:256) showed a survival rate of 80%, from *S. enteritidis* infection. Mice in Group 2 (LPS antibody: titer 1:128) had a 47% survival rate. Mice in Group 3 (Fla antibody: titer 1:1024) had 60%, whereas control mice in Group 4 administered non-immune egg yolk antibodies had the lowest survival rate at 20%. The difference in the survival rates between the OMP antibody group and the control group was statistically significant ($p < 0.05$). The

Table 1 Effect of egg yolk antibody on *in vitro* adhesion of *S. enteritidis* to HeLa cells

Antibody titer		Mean no. of adherent *Salmonella* cells at different antibody dilution					Minimum inhibitory titer[a]
		×10	×20	×40	×80	×160	
OMP	256	6+6[b]**	10+4**	21+7**	23+14**	36+12	3.2
LPS	128	12+8**	17+9**	25+8**	33+12	39+11	3.2
Fla	1024	23+17**	24+8**	33+12	43+15	39+13	51.2
Control	<2	42+12					

[a]Minimum adhesion inhibitory concentration = Antibody titer/Dilution of antibody solution with significant adhesion inhibition
[b]Number of bacteria attached per HeLa cell+Standard deviation
** $p<0.01$ relative to the control (Student's *t* test)

susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. All mice in the control group which survived *S. enteritidis* infection harboured the organism in the liver and spleen regardless of whether excretion in the faeces had ceased or not at the end of experiment. On the other hand, 50% in the OMP group, 86% in the LPS group and 56% in the Fla group were positive for *S. enteritidis* in the liver and spleen organism.

Trial 2. The clinical responses of mice after *S. typhimurium* challenge and subsequent treatment with antibodies are shown in *Figure 2*. Mice in Group 1 (OMP antibody: titre 1:256) showed a 40% survival rate. Mice in Group 2 (LPS antibody: titer 1:128) had a 30% survival rate. Mice in Group 3 (Fla antibody: titer 1:2048) had a 20% survival rate whereas control mice in Group 4 administered non-immune egg yolk antibodies all died. The difference in the survival rates between the OMP antibody group and the control group was statistically significant ($p<0.05$). The susceptible mice manifested the following clinical signs after infection: lethargy, anorexia, and death. On the other hand, 25% in the OMP group, 100% in the LPS group, and 0% in the Fla group were positive for *S. typhimurium* in the liver and spleen organism.

In vitro adherence-inhibition assay

Salmonella adhered to HeLa cells in varying degrees. The average number of adherent bacteria per HeLa cell was 42 for both *S. enteritidis* (*Table 1*) and *S. typhimurium* (*Table 2*). The attachment of bacteria to HeLa cells was reduced by homologous anti-OMP, -LPS, and -Fla antibodies. The minimum antibody titer that significantly inhibited adherence compared to control was 3.2 for OMP, 3.2 for LPS and 51.2 for Fla in the case of *S. enteritidis* homotypic antibodies. For antibodies homologous to *S. typhimurium*, the figures were the same except for LPS antibody which was 6.4.

DISCUSSION

In this report, we examined the effect of oral passive immunization with egg yolk antibodies with specificities against OMP, LPS, and Fla of either *S. enteritidis* and *S. typhimurium* in controlled experimental salmonellosis in mice. The present method of adoptive antibody transfer can serve as a tool to elucidate the importance and role of OMP, LPS, and Fla in the virulence of salmonellae in mice. Particularly, the OMP or porins play a role as pathogenicity determinants. They are exposed on the surface of the bacteria and can interact with cellular and humoral systems[17,38]. The passive transfer of anti-OMP antibodies may therefore influence colonization by *S. enteritidis* and *S. typhimurium* by binding with these surface proteins. Ishibashi *et al.*[21] demonstrated in a mouse model that *Salmonella* OMP induces protection against challenge with *S. typhi*. The OMP produced in that study had 4% LPS contamination and was purified according to the method of Schnaitman *et al.*[35]. Using the anti-OMP antibodies pre-absorbed with LPS, they were able to induce protection in mice.

It appears that aside from microbial characteristics, there are certain host factors which affect the ability of *Salmonella* serotype to survive, multiply, and produce systemic disease. Host factors in adult animals include increased resistance to salmonellosis, attributed to greater immunological maturity of the reticuloendothelial system[39] and microbiological maturity of the gut[40]. The pattern of excretion of *Salmonella* in the faeces of mice between the antibody and control groups was roughly similar within 24 h of oral inoculation of the organisms (data not shown). High viable bacterial counts were detected on day 1 post-challenge and decreased gradually within 72 h. It was during this

Table 2 Effect of egg yolk antibody on *in vitro* adhesion of *S. typhimurium* to HeLa cells

Antibody titer		Mean no. of adherent *Salmonella* cells at different antibody dilution					Minimum inhibitory titer[a]
		×10	×20	×40	×80	×160	
OMP	256	3+4[b]**	9+13**	15+9**	26+10**	36+10	3.2
LPS	128	15+8**	31+12**	38+12	42+11	46+12	6.4
Fla	2048	12+9**	15+7**	27+7**	39+10	43+15	51.2
Control	<2	42+15					

[a] Same as in Table 1
[b] Number of bacteria attached per HeLa cell+Standard deviation
** $p<0.01$ relative to the control (Student's *t* test)

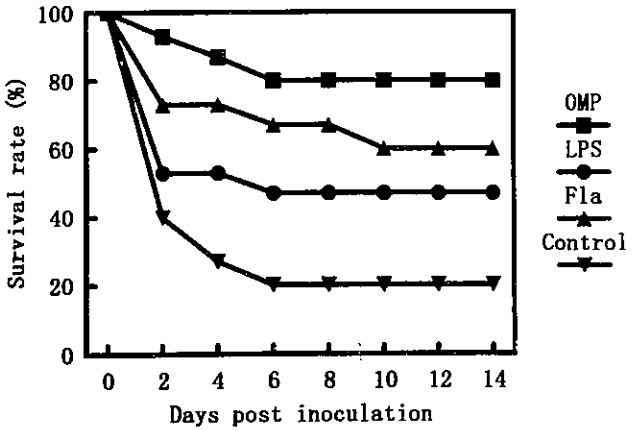

Figure 1 Protection of mice orally administered with egg-yolk antibody to *S. enteritidis*

peak period that the most mortalities were observed in mice (*Figures 1* and *2*). The control mice were continuously shedding *Salmonella* in their faeces, with gradually decreasing viable bacterial counts but evident until two weeks post-infection. Post-mortem bacteriological examination showed various degrees of colonization by *Salmonella* in the liver, spleen, and cecum (data not shown). This suggested a rapid proliferative growth in different organs leading to a systemic disease.

Excretion of *Salmonella* via faeces was eliminated earlier among mice treated with anti-OMP antibodies compared to controls. In the antibody treated group, intermittent excretion of *Salmonella* was generally observed, but viable counts were lower compared to those of the untreated control group.

Salmonella adhered to HeLa cells in varying degrees. The attachment of bacteria to HeLa cells was reduced by homologous anti-OMP, -LPS, and -Fla antibodies. The minimum adhesion inhibitory titers of anti-OMP, -LPS and -Fla antibodies that significantly ($p<0.01$) inhibited adhesion compared to controls was similar for both *S. enteritidis* and *S. typhimurium* (*Tables 1* and *2*). From the data, it appears that anti-OMP had a slight advantage over LPS or Fla specific antibodies.

The difference in survival rates after antibody treatment between *S. enteritidis*- and *S. typhimurium*-infected mice despite similar *in vitro* protection rates by

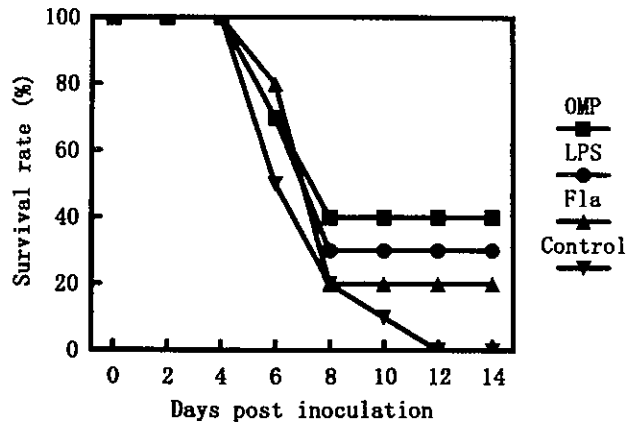

Figure 2 Protection of mice orally administered with egg-yolk antibody of *S. typhimurium*

the antibodies may be attributed to a combination of several factors. *In vitro*, efficacy was judged according to adhesion inhibition only whereas *in vivo*, similar levels of adhesion inhibition may have different colonization outcome due to other factors such as bacterial pathogenecity, challenge dose and host susceptiblity.

The efficacy of anti-OMP egg yolk antibodies can be more appreciated in view of the protection it conferred on mice which appeared to be more susceptible to salmonellosis. The precise mechanisms by which anti-OMP antibodies protect against *Salmonella* invasion of the host is not clearly known. On the basis of the present knowledge on outer membrane proteins of gram-negative bacteria and their biological functions, studies on adhesion to and invasion of the mucosa by *Salmonella*, as well as experimental results from our protection trials, we hypothesize that OMP, since they are exposed on the surface of the bacterial cell, can easily be recognized by antibodies. The subsequent binding may lead to impairment of the biological functions of OMP by disrupting the interaction and presentation of other cell surface components which may also play a role in virulence. The end result is a reduced invasiveness of *Salmonella* with loss of ability to colonize the intestinal tract in numbers sufficient to cause a fatal disease. The use of passively administered egg antibodies may be extended to other species in the form of oral egg antibody preparations.

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