Passive secretory immunity against *Salmonella typhimurium* demonstrated with foster mouse pups

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Summary. Mice immunised by drinking water containing an *Aro*[−] mutant strain of *Salmonella typhimurium* produced intestinal IgA antibodies after a memory response which was demonstrated by measuring copro-antibodies. After oral challenge with a virulent strain of *S. typhimurium*, foster mouse pups placed with immunised mothers survived longer than control pups held with non-immunised mothers.

Introduction

The young of mammals may receive passive maternal antibody prenatally through cross-placental transmission, limited to IgG immunoglobulins, or through yolk-sac absorption; they may also receive it postnatally through colostrum and mother's milk.¹ Ungulates such as cows and pigs receive nearly all of their maternal antibody through colostrum, whereas primates, rabbits and guinea-pigs receive most of their maternal antibody by placental transmission. Rodents and dogs are intermediate, receiving some maternal antibody prenatally but acquiring more postnatally. The ability to absorb from the gut antibody, which appears to be limited to IgG immunoglobulins, and the half-life of passive serum antibody, also vary among young mammals. Gut absorption of antibody stops within 24 h in calves but extends to 14–20 days in rats and mice. Passive immunity in rats and mice due to maternal antibody, declining rapidly about 10 days after birth, has been demonstrated against a variety of micro-organisms.^{1, 2}

It has become increasingly apparent that the secretory immune system plays an important role in host defence against enteropathogenic bacteria, and that vaccination against pathogenic bacteria that colonise or invade through the mucosa is likely to be more effective by oral rather than parenteral routes.^{3, 4} Salmonellosis and typhoid fever are diseases contracted primarily through the gastrointestinal tract. Several types of avirulent mutant strains of *Salmonella typhimurium* have been generated in recent years for studying their potential as vaccines in a mouse model of typhoid fever. Because *Aro*[−] mutant strains such as those developed by Hoiseith and Stocker,⁵ have a requirement for aromatic compounds not found in

mammalian tissue, their capacity for survival *in vivo* is reduced. Mutant strains of this kind have been evaluated as vaccines in several studies.^{6–8}

We describe in this paper the arousal of an intestinal IgA memory response in mice after oral immunisation with an *Aro*[−] mutant strain of *S. typhimurium*, and passive immune protection against oral challenge with *S. typhimurium* in foster pups placed with immunised mothers.

Materials and methods

Bacteria

Two experimental strains of *S. typhimurium* were used in this study. Strain SL3201, a representative of the FIRN biotype, carries a plasmid (60 Mda) associated with virulence, and this strain is highly virulent for BALB/c mice. Strain SL3235, an *Aro*[−] mutant strain derived from strain SL3201, also carries this 60-Mda plasmid associated with mouse virulence (fig. 1) and was received from Professor B.A.D. Stocker, Stanford University, CA, USA.

Experimental strains of bacteria were maintained in peptone 1% w/v containing glycerol 40% v/v at −20°C and subcultured twice in brain heart infusion broth before use. The densities of bacterial suspensions were determined by colony counts on trypticase soy agar containing yeast extract 0.6% w/v.

Plasmids extracted from bacterial cultures by the method of Marko *et al.*⁹ were identified by agar-gel electrophoresis. Reference plasmids were: one of 60 Mda from *S. typhimurium* strain X3306 obtained from Professor R. Curtiss, Washington University, MO, USA; one of 86 Mda from a strain of *Escherichia coli* obtained from Dr Diane Taylor, University of Alberta, Canada; and those of 26, 46 and 72 Mda from strains of *E. coli* obtained from the National Collection of Type Cultures, London.

Fig. 1. Agarose-gel electrophoresis showing plasmids of *S. typhimurium* strains SL3235 and SL3201 (lanes D and E), and mol.-wt reference plasmids of 86 Mda (lanes A and J), 72 Mda (lane I), 60 Mda (lanes C and H), 46 Mda (lanes B and G) and 26 Mda (lane F).

In-vitro interactions between bacteria and epithelial cells

The adhesion and invasion properties of experimental strains of *S. typhimurium* were determined with Henle 407 intestinal epithelial cells in tissue cultures by a method described previously,¹⁰ modified by the inclusion of fixed cells for determining the activity of gentamicin *in situ*. This procedure provides six bacterial counts identified in table I as: (i) "Added," the number of bacteria introduced to each cell monolayer, allowing expression of associated bacteria as a percentage of the inoculum; (ii) "Associated," the total number of bacteria that remain associated after interaction and washing of each cell monolayer, representing extracellular attached and intracellular bacteria; (iii) "Intracellular," the number of bacteria surviving gentamicin treatment for each cell monolayer, representing intracellular bacteria; (iv) "Plastic," the total number of bacteria associated with wells that did not receive Henle 407 cells but were otherwise treated similarly, providing a basis for judging the relative affinity of bacteria for cells compared to that for polystyrene; (v) "Fixed-Associated," the total number of bacteria associated with each cell monolayer that was fixed with paraformaldehyde, representing extracellular attached bacteria only and (vi) "Fixed-Gentamicin," the number of bacteria on each

fixed monolayer that survived gentamicin treatment. The latter two counts are used to support the interpretation that the count of survivors of gentamicin treatment with unfixed cells does in fact represent intracellular bacteria.

Immunisation protocol

Three oral immunisations with *S. typhimurium* strain SL3235 were provided by replacing the drinking water with a suspension of bacteria ($c. 10^8$ /ml in peptone water 0.1% w/v) and given to the mice for 24 h. The first two oral immunisations were 1 week apart, after which immunised female mice were held with male mice for 5–7 days. The third immunisation was administered 19 days after the second, and about 1 week before parturition.

Measurement of copro-antibodies

Faeces were collected periodically from mice by placing them under a beaker on a wire screen lying on the base of a petri dish. The weight of collected faeces was calculated from the gross and tare weights of the petri dish. Faecal material was suspended in 3 ml of buffer (50 mM edetic acid; trypsin inhibitor, 0.1 mg/ml; pH 7.2) and broken up with a glass rod and

vigorous vortex mixing. The volume was made up to 6 ml with phosphate-buffered saline (PBS) and the suspension was centrifuged (850 *g* at 25°C for 10 min). A 4-ml volume of supernate was transferred to a 50-ml conical centrifuge tube and 100 µl of phenylmethylsulphonyl fluoride (PMSF) was added (100 mM in ethanol 95%). The suspension was centrifuged (22 000 *g* at 4°C for 20 min) and 3 ml of supernate was removed for storage at -20°C after addition of PMSF (30 µl), sodium azide 1.0% w/v (30 µl) and fetal bovine serum (150 µl).

Copro-antibodies were measured by an enzyme immunoassay (ELISA) with whole bacteria of *S. typhimurium* strain SL3201 as antigen, suspended at a dry wt of 20 µg/ml in carbonate coating-buffer (0.04 M Na₂CO₃, 0.06 M NaHCO₃, NaN₃, 0.02% w/v, pH 9.6) and added (100 µl) to wells of a 96-well immunoassay plate (Nunc Immuno plate). Plates were held at 4°C overnight, wells were washed three times with PBS (each wash throughout the procedure included 5 min on a rocker), and treated with a blocking buffer (chicken egg albumin 2% w/v in PBS) for 3 h at room temperature. Blocking buffer was removed and plates were stored dry at -20°C.

Wells of antigen plates were washed three times with PBS before use. The sample (serum, or faeces adjusted so that 100 µl of extract represented 3 mg wet weight of faeces), diluted when necessary in buffer (bovine serum albumin 0.1% w/v, BSA, in PBS), was added and the plate held overnight at 4°C. Wells were washed five times with PBST (PBS containing Tween 20 0.05% v/v), and treated with blocking buffer (BSA 1% w/v in PBS) for 1 h. Wells were washed twice with PBST and goat anti-mouse immunoglobulin (IgA, IgM or IgG) (Kirkegaard and Perry) (100 µl) was added at the following dilutions made in buffer (BSA 0.1% w/v in PBST): anti-mouse IgA, 1 in 4000; anti-mouse IgM, 1 in 2000; anti-mouse IgG 1 in 2000. The plate was held at room temperature for 2 h and wells were then washed five times with PBST. Anti-goat IgG-alkaline phosphatase conjugate (Cappel) (100 µl) was added at a dilution which varied with the isotype being measured: IgA, 1 in 6000; IgM, 1 in 4000; IgG, 1 in 4000. The plate was held at room temperature for 90 min, washed five times with PBST, and enzyme substrate [Na₂ *p*-nitrophenyl phosphate, 1 mg/ml in diethanolamine buffer (diethanolamine, 9.7% v/v; 50 mM MgCl₂; pH 9.8)] (200 µl) was added. The plate was sealed and incubated at 37°C by floating in a waterbath for 2 h. The enzyme reaction was stopped by adding 3 N NaOH (100 µl) to each well, and the concentration of chromogen was measured by determining the absorbance at 405 nm with an automated ELISA reader (BioRad).

Each ELISA plate included a negative control consisting of either normal mouse serum or an extract of pooled faeces collected from non-immunised mice, and a positive control consisting of serum from immunised mice or an extract of faeces containing natural IgA against *S. typhimurium* aroused by oral

immunisation, IgG by supplementing with serum from an immunised mouse, and IgM by supplementing with a monoclonal antibody prepared against *S. typhimurium*. The ELISA results for test samples were converted to positive/negative (P/N) ratios, i.e., the ratio of the A₄₀₅ for the test sample to that for the negative sample, as described by Briggs and Skeeles.¹¹ A P/N ratio of 1.0 represents a negative test result. Each assay included triplicate wells with the results converted to the arithmetic mean and standard deviation.

Foster pups

Natural mouse pups from immunised female mice were removed 24 h after birth and replaced by eight pups of the same age taken from non-immunised female mice. Two days later, foster pups and an equal number of control pups left with their natural non-immunised mothers were challenged by oral administration of 20 µl of gelatin 0.1% w/v saline containing *c.* 10³ cfu of *S. typhimurium* strain SL3201. The pups in the test and control groups were checked at least once daily over 22 days to facilitate prompt removal of dead animals and to record the number of days between challenge and death.

Results

In-vitro interactions with epithelial cells

We found that anaerobic growth of strains of *S. typhimurium* increased their invasion of epithelial cells in tissue cultures. Therefore, the adhesion and invasion properties of the two experimental strains of *S. typhimurium* were compared with anaerobically grown cultures. The results (table I) indicated that adhesion and invasion with Henle 407 cells were

Table I. Interactions of anaerobically grown *S. typhimurium* with Henle 407 epithelial cells

Location of bacteria*	Mean (log ₁₀) number of bacteria and standard deviation per monolayer†			
	Strain SL3201		Strain SL3235	
Added	6.42 SD	5.24	6.79 SD	6.27
Associated (percentage of added)	6.05 SD	5.38	5.89 SD	5.65
Intracellular (percentage of associated)	(42.5 SD 10.4)	(12.0 SD 3.2)	5.93 SD	5.37
Plastic (percentage of added)	(75.5 SD 18.2)	(50.7 SD 12.5)	5.63 SD	5.53
	5.37 SD	5.19	4.28 SD	3.27
	(9.0 SD 6.2)	(0.33 SD 0.10)		
Fixed-Associated (percentage of added)	5.70 SD	5.56	4.46 SD	3.94
Fixed-Gentamicin (percentage kill)	(19.4 SD 14.5)	(0.47 SD 0.02)		
	2.54 SD	2.41	1.93 SD	1.65
	(99.91 SD 0.08)	(99.67 SD 0.20)		

* See *Materials and methods*.

† Arithmetic mean and SD for three experiments, each representing composite of three monolayers.

essentially the same for the two experimental strains of *S. typhimurium*. However, for reasons unknown to us, the mutant strain SL3235 showed reduced association for plastic and fixed cells.

Passive immunity in foster pups

Control and foster pups, 24 of each and with each group derived from three litters, were placed with three non-immunised or immunised mothers, respectively. Six of the foster pups were removed from the experiment after 9 days because of the death of one mother. The survival time for 50% of the foster pups placed with immunised mothers was 12 days with three pups still alive after 22 days (fig. 2). In contrast, the survival time for 50% of the control pups was 8 days with none surviving beyond 9 days. This type of passive protection in foster mouse pups was demonstrated in other experiments; however, the data are not presented here because antibody responses were not measured.

Intestinal antibody responses in immunised mice

Immune responses to oral immunisation with *S. typhimurium* strain SL3235 were monitored with five sentinel mice in order to avoid the repeated handling of pregnant females as was required for the collection of faeces. These sentinel mice were immunised and otherwise handled in exactly the same manner as the female mice which had been bred and used for feeding of foster pups. Two baseline measurements were made before the first oral immunisation. There was little or no response for all three isotypes of copro-antibodies against *S. typhimurium* with the first two oral immunisations 1 week apart (figs. 3–7). However, the third oral immunisation, coming 19 days after the second, aroused a memory response that produced large increases in IgA antibody in all five mice. These sentinel mice were synchronised with the pregnant females so that the elevated levels of IgA antibody may be presumed to have been present in

Fig. 2. Survival of foster mouse pups ($n=24$) placed with three mothers immunised by drinking water containing *S. typhimurium* strain SL3235 (●), and natural pups ($n=24$) held with three non-immunised mothers (○), after oral challenge with *S. typhimurium* strain SL3201. Six foster pups were removed at 9 days because of the death of the mother.

the mothers during at least the first 2 weeks of nursing the foster pups. Two sentinel mice also showed increases in faecal IgM antibody following the third booster, but none of the mice showed any increase in IgG antibody.

Humoral antibody responses

Blood was collected from the retrobulbar sinus of the sentinel mice before the first immunisation and at the end of the experiment. Sera were examined by determining isotype-specific ELISA titres against *S. typhimurium* strain SL3201. The results (table II) indicated modest increases in serum IgA and no greater than a four-fold increase in IgG in these sentinel mice. Four of the mice showed high pre-immunisation titres of IgM antibody that dropped sharply. It is known that the rat and mouse fetus synthesise IgM antibody just before birth and that it begins to disappear about 3 weeks later.¹ Pre-immune sera were collected when the mice were about 3 weeks old and this might explain our results; however, it

Fig. 3.

Figs. 3–7. Copro-antibodies against *S. typhimurium* measured by ELISA for sentinel mice, numbers 1–5 respectively, immunised three times (▼) by drinking water with *S. typhimurium* strain SL3235. After the third immunisation, large increases in IgA antibody occurred with mice nos. 1, 4 and 5 (figs. 3, 6 and 7); a large increase in IgA and IgM antibodies occurred with mouse no. 2 (fig. 4); and a large increase in IgA and a smaller increase in IgM antibodies occurred with mouse no. 4 (fig. 5). ↑ = result > indicated value.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Table II. Pre-immune and immune serum titres against *S. typhimurium* for five sentinel mice immunised three times by drinking water containing *S. typhimurium* strain SL3235

Mouse no.	Serum titre*					
	Pre-immune			Immune		
	IgA	IgM	IgG	IgA	IgM	IgG
1	<400	>12 800	1600	3200	1600	3200
2	<400	12 800	800	1600	1600	3200
3	<400	3200	400	400	1600	3200
4	<400	1600	400	400	3200	3200
5	<400	6400	400	400	1600	800

* Highest dilution that gave mean A_{405} ($n=3$) by ELISA that was greater than mean $A_{405} + 2$ SD for negative control ($n=3$).

does not explain why the serum IgM antibody reacted with *S. typhimurium*.

Discussion

Adhesion and invasion of epithelial cells of an animal host are considered basic requisites of bacterial pathogenicity.¹² The most apparent role of mucosal antibodies, especially SIgA, is antigen exclusion.¹³ When these antibodies are directed against bacterial antigens that mediate adhesion or invasion, they may play an important role in preventing colonisation and infection of the host.¹⁴ It was considered important, therefore, to use a vaccine strain of *S. typhimurium* that retained the properties of adhesion and invasion and, presumably, all antigens mediating these processes. We confirmed this for the mutant strain used in this study by showing that its interactions with Henle 407 cells in tissue cultures were essentially the same as those of a highly virulent strain of *S. typhimurium*.

Our main interest in this study was to demonstrate the role of mucosal antibodies, particularly SIgA, in preventing colonisation by *S. typhimurium*. A foster-pup model was chosen because it eliminated any question of protection by antibody received from an immune mother through placental transmission. Culbertson¹⁵ concluded from his study with *Trypanosoma lewisi* that the immunity of normal nursing rat pups placed with an immune mother was as "secure and enduring" as that acquired by the young born of, and nursed by, an immune mother. Bruce-Chwatt and Gibson¹⁶ demonstrated passive immunity against

Plasmodium berghei in baby rats, finding that litters from normal mothers placed with immune mothers showed greater protection than litters from immune mothers placed with normal mothers; this suggested that placental transmission of protection was not as important as that received through mother's milk.

Sentinel female mice were used to demonstrate that oral immunisation through drinking water resulted in the production of intestinal IgA and, accepting the existence of a common mucosal immune system, this antibody should also have been produced in the secretory tissue of the mammary gland.⁴ However, oral immunisation also results in a systemic immune response as was evident from increases in specific IgG antibody which, however, were relatively small (≤ 8 -fold) in the serum of the immunised mice. The suckling foster mouse pups would also have received this antibody and absorbed it from the gut. Therefore, we have not completely resolved with this model the question of how much of the protection observed in foster pups was due to serum antibody acquired by gut absorption of milk antibody, and how much was due to intestinal IgA antibody which is confined to the gut.

The foster mouse-pup model suffered in one additional respect and that was the eventual death of many of the test animals with very few surviving the duration of the experiment. We believe this resulted from repeated exposure caused by infection of the mothers. Again, we believe that the mothers, although immunised, developed infections because of massive doses of bacteria received through ingestion of pup faeces, which they normally do in cleaning the young, and particularly through cannibalism. Mothers that died during the course of this and other experiments were found to have massive systemic infections, and even some that survived were found to be colonised with *S. typhimurium*. The mice were held on wire to reduce coprophagy, the cages were changed every second day, and pups were checked at least twice daily to remove dead animals, all of which did not prevent this problem. Nevertheless, the experimental results did demonstrate significant differences in survival times between foster pups placed with immunised mothers and pups held with their natural non-immunised mothers.

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