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The protection of infant mice from colonization with *Campylobacter jejuni* by vaccination of the dams

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SUMMARY

Intraperitoneal vaccination of female mice, before mating, with a whole cell, heat-killed (62 °C) vaccine of *Campylobacter jejuni* allowed the mother to confer immunity to her young, challenged orally 4–6 days after birth with the homologous strain. There was no protection against a strain of another serotype. Heating the vaccine to 100 °C destroyed its protective properties. A vaccine prepared from an aflagellate variant of the original strain was as protective as the original vaccine against challenge with the flagellated strain. Anti-flagellar serum antibody titres of the dams did not correlate with protection of their young.

INTRODUCTION

The mechanisms by which *Campylobacter jejuni* causes diarrhoea in man are still unknown. The organisms colonize the colon (Lambert *et al.* 1979; Price, Jewkes & Sanderson, 1979) when the pathology is a colitis. However, the jejunum and ileum may also be infected (Blaser & Reller, 1981) particularly in immunosuppressed patients and sometimes without the organisms being excreted in the stool (Ward, Klein & Borthistle, 1984; Lever *et al.* 1984).

Many intestinal pathogens have been shown to adhere to intestinal mucosa as a preliminary to causing disease. Campylobacters have been demonstrated on human colonic biopsy tissue by immunofluorescent staining (Price *et al.* 1984). In other bacteria, pili and flagella contribute to the properties of adherence and invasiveness. *C. jejuni* does not possess pili to aid in attachment (Dijs & de Graaf, 1982) but the single, bipolar flagella are prominent and up to 4 μm long (Pead, 1979). The presence of flagella rather than the property of motility is important in the colonization of the mouse gut (Newell, McBride & Dolby, 1985) and an adhesin on flagella for an intestinal cell line has been demonstrated for *C. jejuni* (McBride & Newell, 1983; Newell, McBride & Dolby, 1985).

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Since flagella comprise the major cross-reacting surface antigen(s) in naturally-aquired infection in humans (Newell, 1983; Wenman *et al.* 1985) they have been proposed as possible vaccine candidates: immunity to flagella may help to prevent colonization of the gut. We therefore embarked on active immunization experiments, in the infant mouse model (Field *et al.* 1981), with vaccines made from flagellated and non-flagellated organisms. The normal course of a 3-week infection in infant mice has been described previously (Newell & Pearson, 1984). Preliminary investigations indicated that intraperitoneal vaccination of dams before mating conferred protection on their young against such infection, either reducing colonization or preventing it altogether. Colonization has now been measured in their offspring and compared with those of control young of non-vaccinated dams challenged orally 3–6 days after birth.

METHODS

Mice

Female Balb/c mice 4–6 weeks old were given 0·2 ml vaccine intraperitoneally once weekly for 4 weeks. They were mated within 4 days of completion of the course to male mice of about the same age, one male being caged with 3–4 females. Pregnant mice near term were caged separately and birth dates noted. The infant mice were challenged orally 4–6 days after birth.

Vaccines

Two strains of *C. jejuni* (flagellate and aflagellate) were each used to prepare three types of vaccine: whole cells heated to 62 °C; whole cells heated to 100 °C; outer membrane protein extracts. The strains were *C. jejuni* 81116 (NCTC11828) isolated from an outbreak of diarrhoea in a school (Newell *et al.* 1985) and its aflagellate variant SF-2 (NCTC 11827) derived as described (Newell, McBride & Pearson, 1984). The original strain is serotype 6 by both its heat-stable (Penner & Hennessy, 1980) and heat-labile (Lior *et al.* 1982) antigens (PEN 6, LIO 6). The strains were harvested into saline from 24–48 h blood agar cultures, incubated at 37 °C in microaerobic conditions, and heated at 62 °C for 45 min or in a boiling water bath for 1 h. Suspensions were adjusted to 10 or 50 International Opacity Units (i.o.u.). Enough vaccine was made for a course of injections to one batch of mice and stored for up to 6 weeks at 4 °C.

Outer membrane protein (OMP) vaccines were prepared from strain 81116 and its aflagellate variant by sarkosyl extraction of crude membranes (Newell, McBride & Pearson, 1984) and injected in 50 *µ*g doses.

Challenge strains

Infant mice were challenged with flagellated strain 81116 (the strain from which the vaccines were made) or 53729/80 (NCTC 11626) isolated from a patient at Northwick Park Hospital who subsequently developed arthritis. This strain was serotype PEN 27 LIO 23. Strains were harvested into Brucella broth (Difco Laboratories Ltd) containing campylobacter supplement SR84 (Oxoid Ltd) from 20–24 h blood agar cultures incubated microaerobically at 37 °C. Suspensions were adjusted to an opacity of 20 i.o.u. and diluted 1/2 in sterile skimmed milk. Infant

mice were given an oral dose of about 0·02 ml (about 1×10^7 c.f.u.) from a 25 G X 5/8 needle capped with narrow polythene tubing (800/100/140. Jencons H64/50). Previous studies showed that both strains colonized infant mice for up to 21 days or longer (Newell, McBride & Dolby, 1985).

Determination of the degree of protection against colonization

Infant mice were killed by cervical dislocation at intervals up to 10 days after challenge. Small segments of colon about 5 mm long were taken from about 2 cm above the anus and homogenized in 1 ml of supplemented Brucella broth. Viable counts were estimated on blood agar plates (2 % agar) with and without Skirrow's selective antibiotics (Oxoid, SR69). Counts were expressed per 1/50 specimen (numbers in 0·02 ml), which was equivalent to $n \times 10^4$ per gram of tissue.

In each family two infant mice were killed on each of 2 days between 2–10 days after challenge. Results were expressed as a dam protecting or not protecting her family, and were dependent on determinations from four infants, except rarely in the case of small families. The dam was regarded as protecting her young completely if all were non-colonized on both occasions or if the average count was 10000 times less than that of the controls. The family was regarded as non-protected if the count from all infants was not reduced to 100 times below that of the controls. Partial protection was accorded to anything in between . An example of the results from one experiment can be seen in Table 1. Numerical values were accorded to these degrees of protection: protected families, 1; partially protected families, 0·5; non-protected families, 0.

Anti-flagellar titres of maternal sera

At about 3 weeks after challenge of their young the dams were bled under anaesthesia. Sera were separated and stored at −20 °C until assayed.

Flagella preparation. Flagella were isolated from *C. jejuni* strain 8116 by shearing and purified by differential centrifugation as previously described (Newell, McBride & Pearson, 1984). The purity of the preparation was checked by electron microscopy of negatively-stained preparations and by SDS-polyacrylamide gel electrophoresis.

Enzyme linked immunosorbant assay. Volumes of 100 μl per well of purified flagella (1 μg/ml) in 0·05 M carbonate buffer, pH 9·6, were incubated for 18 h at room temperature in flat-bottomed micro-ELISA plates (Dynatech Laboratories). The wells were washed with ELISA wash (0·85 % sodium chloride containing 0·05 % Tween 20) then incubated with 100 μl of diluted mouse sera for 2 h at 37 °C. After washing, the bound antibody was detected by incubation with 100 μl of rabbit anti-mouse IgG conjugated to peroxidase (Miles Research Laboratories) (1/1000 dilution in ELISA wash containing 1 % bovine serum albumin and 0·5 % TRIS pH 7·6) for 2 h at 37 °C. The bound peroxidase was detected with a substrate containing 10 μg o-phenylenediamine dissolved in 1 ml of warm methanol and diluted to 100 μl with water containing 10 μl hydrogen peroxide (100 vol). 100 μl of this substrate was added to each washed well and incubated for 1 h at 37 °C in the dark. The optical density was determined at 490 mm in a micro-ELISA reader (Dynatech Laboratories).

Table 1. *Numbers of* C. jejuni *recovered from colonic tissue of infant mice of five vaccinated and one non-vaccinated dams given the same oral challenge with the homologous strain*

| | Serial no. of dam | Viable organisms* | | Protection | |
		Day 2	Day 8	grade	Numerical equivalent
Vaccinated	1	0, 0†	0, 0	+	1
	2	0, 0	0, 2×10^1	±	0·5
	3	3×10^2, 3×10^2	1×10^1	−	0
	4	0, 0	0, 0	+	1
	5	5, 3×10^2	0, 0	±	0·5
					3/5
Non-vaccinated	6	4×10^1, 3×10^1	5×10^1, 1×10^5	−	0

* from 1/50 of specimen (see Methods).
† < 50.
+ colonization by *C. jejuni* 10000 times less than control; organisms not isolated.
± colonization by *C. jejuni* 100 times less than control.
− colonization similar to controls (young of non-vaccinated mothers).

Table 2. *Comparison of 81116 flagellated and non-flagellated vaccines in dams and their ability to protect the young against oral infection with strain 81116*

| | No. families protected | | | | |
| | − | ± | + | Numerical | Protection |
Vaccine*	0	0·5	1	value	%
81116 (Fla⁺) heated 62 °C	16	4	8	10/28	35
SF-2 (Fla⁻) heated 62 °C	19	2	13	14/34	41
None	26	1	0	0·5/27	2

* 0·2 ml of vaccine at 10 i.o.u. was injected intraperitoneally four times at weekly intervals.

RESULTS

The effect of maternal vaccination on protection

In general, effectively protected infant mice were consistently not colonized by *C. jejuni*, with complete absence of organisms or very low numbers. Non-protected mice were consistently colonized to about the same extent as each other. Examples of *C. jejuni* counts for one challenge of six dams, five vaccinated with 10 i.o.u. and one non-vaccinated are given in Table 1 with the assessment of protection accorded to each. The young of dam No. 2 (Table 1) illustrates the assessment of an infrequently occurring inconsistent degree of protection, only one of four infants being colonized, which reduced the protection score by that dam to a partial one as shown.

The role of flagella in the vaccine

The effect of flagellated and non-flagellated vaccine (10 i.o.u.) was compared in 62 vaccinated families and the accumulated results of 5 batches of paired vaccine in 5 batches of mice given in Table 2. For 3 of the 5 batches protection was better

Table 3. *Serum antiflagellar titres of vaccinated dams 3 weeks post partum and the degree of protection conferred on challenged young*

Cage No.	Vaccine (8116 or SF-2)	Maternal anti-flagella titre* ELISA	Protection†
64	Fla$^+$	3400	±
65	Fla$^-$	0	+
96	Fla$^+$	2600	−
97	Fla$^-$	0	+
74	Fla$^+$	6400	
75	Fla$^-$	303	
88	Fla$^-$	0	Young not challenged
Normal pool	None	0	

* Obtained by extrapolation of the linear portion of the serum dilution *vs* OD$_{492}$ curve to OD$_{492}$ = 0·1.
† See Table 1.

Table 4. *The effect of increasing vaccine dose on the ability of dams to confer immunity on young against oral challenge with strain 81116*

Vaccine*	Dose	No. families protected			Protection %
		−	±	+	
Fla$^+$ 62 °C	10 i.o.u.	3	3	0	25
Fla$^+$ 62 °C	50 i.o.u.	0	3	4	79
Fla$^-$ 62 °C	10 i.o.u.	4	2	2	38
None	—	7	0	0	0

* 0·2 ml intraperitoneally, four times at weekly intervals of 81116 vaccines.

with the aflagellate than with the flagellate vaccine; for 2 batches the flagellated was better than the aflagellated vaccine giving an overall slight advantage to the latter. We consider the results with the two vaccines were not significantly different.

The anti-flagellar titres of maternal mouse sera are given in Table 3. It can be seen that the ability of a dam to protect her young was not dependent on a high titre of anti-flagellar antibodies as measured by the enzyme-linked antibody assay.

Effect of increasing the strength of the vaccine

Increasing the dose of vaccine from 4 doses of 10 i.o.u. to 4 doses of 50 i.o.u. increased the degree of protection accorded to the young by the vaccinated mothers. This is demonstrated for one batch of vaccine of the flagellated strain in Table 4.

Effect of boiled and outer membrane protein vaccines

Table 5 illustrates the lack of protection conferred by a non-flagellate vaccine heated for 1 h at 100 °C compared with the 40 % protection of the 62 °C heated vaccine.

Table 5. *Comparison of different vaccine preparations of* C. jejuni *81116 or SF-2 given intraperitoneally to dams in conferring protection on orally challenged young*

	No. families protected			Protection
Vaccine*	−	±	+	%
Fla⁻ 62 °C (10 i.o.u.)	3	5	2	40
Fla⁻ 100 °C (10 i.o.u.)	12	0	0	0
OMP Fla⁻ (50 μg)	5	(2)†	0	10
OMP Fla⁺ (50 μg)	7	(2)†	0	8
None	10	0	0	0

* 0·2 ml, four times at weekly intervals.
† Families colonized slightly more than definition ± in Table 1; i.e. protection very weak.

Table 6. *Numbers of vaccinated families protected against challenge with homologous and heterologous strains*

Challenge strain	81116 Fla vaccine			SF-2 Fla vaccine			Non-vaccinated		
	−	±	+	−	±	+	−	±	+
81116	3	2	0	0	0	3	3	0	0
53729	6	0	0	3	0	0	3	0	0

Outer membrane protein vaccine (four doses of 50 μg each) was non-protective.

The strain specificity of protection

The protection by 81116 vaccines (Fla⁺ and the SF-2 non-flagellated variant) against the homologous, flagellated strain 81116 was compared with protection by the same vaccines against a flagellated strain of another serotype, 53729. There was none against the heterologous challenge as shown in Table 6. Both challenge strains were similarly virulent. Thus a vaccine of serotype PEN 6; LIO 6 does not protect against a challenge with serotype PEN 27; LIO 23.

The mice used in this experiment were much better protected by the SF-2 than flagellated vaccine. The figures have already been included in Table 2 and exemplify one of the three vaccine batches in which protection was better with the aflagellate vaccine.

DISCUSSION

The results presented in this paper demonstrate that infant mice can be protected against intestinal colonization from an oral challenge with *C. jejuni* by immunizing the dams intraperitoneally with whole-cell vaccines subjected to heat sufficient to kill the campylobacter (62 °C). Vaccine made from a strain of serotype 6 protected mice against challenge with the homologous strain but not against a strain of another serotype. We did not determine if protection would be afforded to another strain of serotype 6. The serotype specificity of the Lior scheme (Lior *et al.* 1982) is probably due to flagella: the aflagellate strain SF-2 was non-typable by the Lior scheme but protective as a vaccine. Lipopolysaccharide, also conferring

serotype specificity (Penner & Hennessy, 1980), is stable to 100 °C (Logan & Trust, 1984; Naess & Hofstad, 1984), but vaccine heated at 100 °C was ineffective.

The presence of flagella plays an important part in the ability of *C. jejuni* to colonize the infant mouse gut (Newell, McBride & Dolby, 1985). Slight but definite protection was afforded to challenged infant mice injected with mouse antiserum to purified flagellar protein (Newell, unpublished). Attridge & Rowley (1983) also showed that in *Vibrio cholerae* the flagella are important in aiding colonization by adherence. Our active immunization results demonstrate clearly, however, that the presence or absence of flagella in the strain from which the vaccine is made is irrelevant to the production of maternally transmissible immunity in the mouse model. There was only a slight difference between the protective capacity of vaccines made from flagellated and non-flagellated bacteria (36 and 41 % respectively) and these are not considered significant. All batches of vaccine were checked by electron microscopy to ensure that flagella were not present in the aflagellate vaccine. Confirmation was provided by the titres of anti-flagellar antibody in the sera of vaccinated dams.

Findings for *C. jejuni* are unlike the apparent situation in cholera, in which a crude flagellar preparation protected rabbits (Yancy, Willis & Berry, 1979). The authors considered the possibility, however, that the active vaccine component was a heat-labile bacterial cell surface component other than flagella. Surface adhesins other than flagella may also be expressed by *C. jejuni* (Newell & McBride, 1983). The relevance of these adhesins in virulence has yet to be established.

The vaccine dose used for most of the experiments (10 i.o.u.) was around the PDso value where small variations in potency had a large effect on percentage protection. Increasing the vaccine dose five times doubled the percentage protection. The relevant antigen may not be particularly stable or immunogenic, perhaps because of the 62 °C heat-treatment. Experiments are in progress to determine whether unheated vaccine has a greater effect (Hassan, personal communication). Preliminary experiments have shown that some protection is conferred on the litters of mice previously exposed, either as nursing mothers or as babies, to live organisms administered orally. The routes of vaccination and the transfer of protection are also under investigation.

Although we were not able to define the protective component of a *C. jejuni* vaccine, we have shown that immunization in the mouse model can prevent colonization. The protection of rhesus monkeys from rechallenge (Fitzgeorge, Baskerville & Lander, 1981) and the high frequency of carriage in children continually exposed to infection in developing countries (Bokkenheuser *et al.* 1979; Rajan & Mathan, 1982) suggest that therapeutic immunization will be possible in primates, in whom the response to infection is more severe than in mice. The course of infection in healthy individuals is self-limiting, but hypogammaglobulinaemic patients who become colonized need help in overcoming the infection. It is for these patients especially that a clearer understanding of protective factors would be of practical benefit.

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