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Comparison of different vaccines and induced immune response against *Campylobacter jejuni* colonization in the infant mouse

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SUMMARY

The degree of protection conferred by vaccinated dams on infant mice against colonization by *Campylobacter jejuni* depended on the bacterial strain, preparation, and route of administration of the vaccine. In some instances of homologous protection, serum bactericidal titres correlated well with protection. However, boiled *C. jejuni* vaccine, which was non-protective, also elicited a strong bactericidal antibody response. Conversely, bactericidal activity could not be demonstrated against strains capable of cross-protection. There was a good correlation between high campylobacter-specific IgG response and bactericidal activity.

INTRODUCTION

The immunological mechanisms underlying recovery from bacterial gut infections are largely unknown. However, a major role has been postulated for secretory antibody in preventing the establishment of infectious agents (Tomasi *et al.* 1965). Non-immunological defence, which includes epithelial cell-integrity, enzymatic activity, gastric acidity and gut motility may also be involved. Often these various systems together with the immune system are considered to be the main mechanisms of mucosal protection (Waldman *et al.* 1971; Chipperfield & Evans, 1975). *Campylobacter jejuni* enteritis occurs in immunodeficient patients with generalized hypogammaglobulinaemia (Lever *et al.* 1984), or with specific decrease in IgA or IgM antibodies (Glover *et al.* 1982; Johnson *et al.* 1984; LeBar *et al.* 1985), frequently leading to recurrent bacteraemia and sometimes chronic diarrhoea.

There is little known about mechanisms involved in protection against *Campylobacter jejuni* but a potent bactericidal activity in human and rabbit sera against *C. jejuni* has been reported (Blaser *et al.* 1979, Border *et al.* 1974). Pennie and co-workers (1986) demonstrated a strain-specific bactericidal activity to *C. jejuni* in sera of infected patients which seemed to be dependent on specific IgM. In addition, prolonged bacteraemia and systemic infections due to *C. fetus* has been associated with its resistance to serum bactericidal activity (Blaser *et al.* 1985).

We have investigated the prevention of colonization of the intestinal tract of infant mice by *C. jejuni* and found that this could be prevented by vaccination of

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the dams with the challenge strain prior to mating (Dolby & Newell, 1986). This homologous protection, and cross-protection which was first demonstrated by us among *C. jejuni* strains with the same Lior serotyping antigens (Abimiku & Dolby, 1988), was dependent on immune milk taken at and after oral challenge (Abimiku & Dolby, 1987) and provided material for correlation of *in vitro* and *in vivo* antibacterial activity.

Preliminary experiments showed that mouse milk or sera were unable to prevent adhesion of *C. jejuni* to intestinal tissue cell lines, and that lactoferrin or transferrin mediated bacteriostasis did not occur (unpublished). This paper explores first the influence of vaccine preparation and dose, route of administration, and vaccine strain on protection; and second, the relationship between bactericidal activity of milk and sera of vaccinated mice and the ability of those mice to protect their infants against colonization.

MATERIALS AND METHODS

Bacterial strains. Strains of *C. jejuni* used were the wild-type strain 81116 (NCTC 11828, National Collection of Type Cultures, Colindale Avenue, London NW9 5HT) described elsewhere (Newell *et al.* 1985). This strain colonized well and made a good vaccine. It gave rise to an aflagellate variant-SF2 (NCTC 11827) derived as described by Newell *et al.* (1984) which does not colonize infant mice but which also makes a good vaccine (Dolby & Newell, 1986). The original wild type strain 81116 is serotype 6 by both its heat-stable (Penner & Hennessy, 1980) and heat-labile (Lior *et al.* 1982) antigens, expressed as PEN 6 LIO 6. Other strains used were those matched to strain 81116 either by their L10 antigens (strains 13024, PEN 29 LIO 6; strain 608, PEN 7 LIO 6; strain CCUG 12066, PEN 25 LIO 6) or by their PEN antigens (strain 20186, PEN 6 LIO 11); strain 53729 (NCTC 11626), PEN 27 LIO 23 described in detail previously (Dolby & Newell, 1986) matched for LIO or PEN serotyping antigens with strains 18203, PEN 27 LIO 11 and strain CCUG 15023, PEN not-typable (NT) LIO 23.

Strains 12066 and 15023 were from the Culture Collection, University of Goteborg (CCUG), Sweden, and other non-NCTC strains from the Public Health Laboratory, Withington Hospital, Manchester.

Vaccine preparation. All killed vaccines were prepared by harvesting 24–48 h growth off two blood agar plates into 10 ml phosphate-buffered saline (PBS) pH 7·2 and heating to 62 °C for 45 min or 100 °C for 1 h. In other instances, the harvested growth was also killed by mixing with 10 ml of formal saline with 0·5% v/v formalin and holding at 4 °C for 1–2 days, centrifuging and resuspending in PBS. Opacities were then compared by eye and adjusted against the International Standard (National Institute for Biological Standards and Control) to 5, 10 or 50 International Opacity units (IOU) as appropriate. Vaccine was stored at 4 °C over the 1-month period of vaccination.

Live vaccine was made from a loopful (1 μl, NUNC) of a 24 h growth of *C. jejuni* harvested into 10 ml of PBS pH 7.2, standardized to 5 IOU and used within 1 h (Abimiku & Dolby, 1988).

Vaccination. Female Balb c mice were injected intraperitoneally with 0·2 ml of killed or live vaccine once weekly for 4 weeks at concentrations of 5, 10 or 50 IOU. Oral vaccination was administered from a syringe attached to a needle capped with

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narrow polythene tubing (800/100/140, Jencons H64/50) to adult mice in 0·2 ml volumes containing 10^9 colony-forming units (c.f.u.) once weekly for 4 weeks. Faecal pellets collected routinely from mice vaccinated with the live vaccines, were resuspended in Brucella broth and cultured for *C. jejuni*.

Females were mated by caging three females to a male within 24 h of completion of vaccination and the young were delivered to individually caged dams 3 weeks later.

Assay of vaccine potency. Infant mice, 4–6 days old, were challenged from a capped syringe with 0·02 ml of a suspension of overnight growth of *C. jejuni*, made to 20 IOU and diluted in an equal volume of sterile skimmed milk, containing about 10^7 viable *C. jejuni* as described previously (Dolby & Newell, 1986), and the degree of colonization determined, and protection recorded. Briefly, a segment of the colon (approx. 5 mg) was homogenized and serially diluted in tenfold dilutions (10^{-1} to 10^{-3}) in Brucella broth (Difco) before inoculating 20 μl onto Skirrow's campylobacter-selective medium and non-selective media (Columbia blood agar by Oxoid) and incubated. A reduction of 100-fold or more of *C. jejuni* c.f.u., compared with the average for infants of non-vaccinated dams, was considered evidence of protection.

Collection of serum and milk. Serum was collected from vaccinated dams about 3 weeks after delivery (Abimiku & Dolby, 1987). Infant stomachs containing milk were removed about 7 days after delivery. These and earlier milk and sera were kept at −70 °C. When required, each stomach containing mouse milk was homogenized in four volumes of PBS and ultracentrifuged at 12000 g for 3 min and the supernatant retained.

Antibody determination by ELISA. At least five individual specimens of sera and milk collected from vaccinated dams for each vaccine, were tested for the presence of *C. jejuni* specific antibodies by the enzyme-linked immunosorbent assay (ELISA). Briefly, each well in an ELISA microtitre tray (Nunc) was coated overnight at room temperature with 100 μl of a supernatant of sonicates of mixed *C. jejuni* vaccine strains 81116, 20186 and 53729 containing 10 μg/ml of protein. Mouse serum was diluted (1:2000) in ELISA diluent (1% bovine serum albumin (BSA) in 0·6% TRIS pH 7·6). Mouse milk prepared in PBS (see above) was further diluted by 1:40 to give a start dilution of 1:200. Both samples were then diluted individually in twofold dilutions in ELISA diluent. Milk and serum from non-vaccinated dams were used as negative controls at a dilution of 1:200, and campylobacter specific IgA monoclonals was used as a positive control for specific IgA assay at a dilution of 1:6000. The tray containing bound antigen was washed three times with ELISA wash (PBS/tween, Don Whitley Scientific Ltd, Shipley, West Yorkshire BD127 5JS). Aliquots of 100 μl of dilutions of serum or milk were allowed to react with the bound antigen for 2 h, followed by a washing step and the addition of anti-mouse polyclonal (whole molecule), IgG (γ-chain specific), IgA (α-chain specific), secretory IgA (whole molecule) or IgM (μ-chain specific) alkaline phosphatase conjugate (Sigma Ltd). The tray was again washed three times and the bound enzyme detected by the addition of the substrate (a disodium salt of *p*-nitrophenyl phosphate tablet dissolved in 10% diethanolamine buffer, pH 9·8 (Don Whitley Scientific Ltd)). The optical density was read on a microELISA reader at 405 nm.

Bactericidal test. Test *C. jejuni* strains were vaccine strains 81116, 20186 and

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Table 1. *Protection by intraperitoneal vaccination of live or killed* C. jejuni *strains: homologous challenge*

Vaccine	Vaccine concentration	Litters	No. protected/no. challenged (%)
None	—	3	0/12
81116			
Live	5	3	8/8 (100)
Formal saline	50	3	8/12 (67)
62 °C	50	8	22/32 (71)
100 °C	50	3	0/9
81116 Fla⁻ †			
Live	5	3	7/9 (78)
62 °C	50	3	9/12 (75)
20186			
62 °C	50	3	3/10 (30)
53729			
62 °C	10	4	10/11 (91)

* Expressed in international opacity units (IOU).

† Aflagellate variant derived from strain 81116. The challenge was 10^7 c.f.u. wild-type strain 81116.

53729, and strains matched to them by their L10 or PEN serotype antigens (listed in Table 3). They were grown on blood agar microaerobically for 18–24 h and harvested into Brucella broth (Difco). Suspensions at 5 IOU were further diluted 1:1000 in Brucella broth for the test.

Guinea-pig serum (source of complement) stored frozen at −70 °C was thawed and diluted 1:8 in PBS. The milk supernatant, and thawed serum diluted 1:10, were heated at 56 °C for 30 min. For the test, 20 μl of diluted milk or serum and 20 μl of diluted guinea-pig serum were added to 20 μl of the bacterial inoculum. Serum, milk, complement and organism controls were all set up in parallel. Trays were incubated microaerobically at 37 °C for 30 min, then three tenfold dilutions from each well were made in Brucella broth and inoculated on blood medium with 2% agar to inhibit swarming. Plates were incubated for 2 days microaerobically and viable organisms estimated. The percentage decrease in the number of organisms in the test wells, compared to the control wells with organisms only was recorded as evidence of bactericidal activity.

RESULTS

Importance of vaccine preparation in protection

Experiments involving the intraperitoneal (i.p.) injection of 0·2 ml of live vaccine at 10 IOU prevented pregnancy after mating, either due to lack of conception or due to the resorption of the foetuses. However, a live vaccine at 5 IOU allowed successful pregnancies. Killed vaccines were given at 10 or 50 IOU as indicated (Table 1).

The method of measurement of protection has made it impossible to carry out extensive PD_{50} determinations but our results have been consistent within batches

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over numerous tests. The protection determined by viable count estimations of orally challenged infants of dams immunized with live, heated, or formalin-killed wild-type strain 81116 and the aflagellate variant of 81116 (Fla^-) are summarized in Table 1.

Live vaccine made from the wild-type strain 81116 was the most effective, protecting all infant mice from colonization by *C. jejuni*. Heated (62 °C) and formalin-treated vaccines were less so, and required a more concentrated ($\times 10$) vaccine preparation. Boiled (100 °C) vaccine did not protect any of the challenged infants even at the higher concentration. Vaccine from the aflagellate strain gave the same pattern of results. In general, infants of non-vaccinated dams were consistently colonized by *C. jejuni* to about the same extent, but slight variation was found among infants of vaccinated dams.

Importance of route of vaccination in protection

The oral route, which is the natural route of infection, was compared with the i.p. route, for live vaccine of strain 81116 at a concentration of 5 IOU. The live vaccine protected 23 of 24 (96%) infant mice derived from 6 litters when given i.p., but only 4 of 14 (29%) derived from 3 litters when given orally, in homologous challenge experiments. *C. jejuni* was cultured from the faecal pellets of adult mice during, and up to 2–3 weeks after, i.p. but not oral vaccination with the live vaccine.

Importance of vaccine strain in protection

A comparison of heated (62 °C) vaccines made from different strains and administered i.p. showed the best homologous protection for strain 53729 even though it was used at the lower concentration of 10 IOU, and the poorest for strain 20186 (Table 1). Vaccines prepared from killed strains 81116 and 20186 were not effective at 10 IOU (data not shown). The results illustrate considerable strain differences in the ability of *C. jejuni* strains to make good vaccines.

Antibodies in mouse milk and serum

High titre of campylobacter specific IgG antibodies was present in the serum (32000–64000) and milk (16000–32000) of mice vaccinated intraperitoneally with live, formalin-killed, or heated (62 °C) suspensions of *C. jejuni* (Table 2). Mice immunized orally with live *C. jejuni* or vaccinated i.p. with boiled vaccine had low specific IgG in their serum (8000–16000) and milk (400–800). Campylobacter specific IgA, sIgA, or IgM antibodies were not detectable in the serum and milk of vaccinated dams. However, control wells with campylobacter-specific IgA monoclonals were all positive. The serum and milk from non-vaccinated dams had no detectable campylobacter-specific antibodies.

Bactericidal activity and protection

Bactericidal activity was measured in at least five individual specimens of serum and milk for each vaccine. Bactericidal activity against the homologous strain was evident to the same degree in milk and serum from dams vaccinated i.p. with treated or live vaccines, irrespective of the efficacy of the vaccine in protection (Table 2). However, the oral live 81116 vaccine stimulated low

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Table 2. *Immune response, bactericidal activity and protection against the homologous strain following vaccination*

Vaccine (i.p.)	Bactericidal activity*		ELISA antibodies†		Protection†
	Serum (1:10)	Milk (1:5)	Serum	Milk	
81116					
Live	100	100	32000	1600	100
FS§	100	100	32000	1600	67
62 °C	100	100	32000	1600	70
100 °C	100	100	1600	800	0
81116 Fla⁻ ‖					
Live	100	100	32000	1600	78
62 °C	100	100	32000	1600	75
53719					
62 °C	100	100	32000	1600	91
20186					
62 °C	90	89	32000	1600	30
81116 (oral)					
Live	0	0	8000	400	29

* % Decrease in log c.f.u. over 30 min-period from an initial 10^7 c.f.u. using an average of five different specimens for each test.

† Reciprocal titres.

‡ Data from Table 1 and text.

§ Formal saline treated.

‖ Aflagellate variant from strain 81116.

Table 3. *Bactericidal activity and cross-protection against the heterologous strains following intraperitoneal vaccination*

Vaccine (i.p.)	Test strain	Bactericidal activity (%)*		Protection (%)
		Serum (1:10)	Milk (1:5)	
81116 PEN 6 LIO 6	81116	100	100	100
	13024 PEN 29 LIO 6	0	0	43†
	608 PEN 7 LIO 6	0	0	N.D.
	12066 PEN 25 LIO 6	70	65	64†
	20186 PEN 6 LIO 11	0	0	0†
53729 PEN 27 LIO 23	53729	100	100	92
	15023 PEN N.T. LIO 23	0	0	77†
	18203 PEN 29 LIO 11	0	0	0†

* Bactericidal activity in milk and serum estimated as in Table 2; ELISA titres were also the same in Table 2.

† Percentages from Abimiku & Dolby, 1988.

N.D., not done. N.T., not typable.

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antibody levels, and did not induce bactericidal activity. Furthermore, except in one case, bactericidal activity could not be shown against heterologous strains matched to the vaccine strain by either of the serotyping schemes (Table 3). The close similarity between vaccine strain 81116 and L10 matched strain 12066, indicated by the better cross-protection achieved against that strain than any other L10 matched strain (Table 3), has been reflected in the bactericidal activity by anti-81116 milk and sera against strain 12066 (Table 3).

DISCUSSION

The general findings that live vaccine given intraperitoneally gave good protection against colonization by *C. jejuni*, whereas vaccine heated to 62 °C protected less well and boiled vaccine not at all, imply that heat-labile antigens may be important. Although, it is possible that the prolonged antigenic stimulation due to subsequent establishment in the gut following live vaccination, also played a role. However, vaccines were effective only when given intraperitoneally and not orally, and there was strain-to-strain variation, with one strain (53729) giving good protection at relatively low concentrations following heating at 62 °C and another (20186) giving poor protection even at relatively high concentrations.

The presence of specific IgG immunoglobulins and the lack of specific IgA immunoglobulins in the milk of vaccinated dams was rather unexpected since parenteral immunization with other enteric organisms had previously led to production of a specific IgA as well as IgG response (Keren *et al.* 1983). The ELISA assay used in this study detected low amounts of specific IgA immunoglobulins in the positive control wells, which seems to indicate a true absence of specific IgA immunoglobulins in the milk of vaccinated dams. Although this phenomenon, i.e. the lack of specific IgA antibodies in the presence of specific IgG antibodies in the milk of immune animals, is unexpected, it is not uncommon. Theodore *et al.* (1982) demonstrated only specific IgG antibodies in the colostrum and milk of rabbits immunized intravenously with respiratory syncytial virus. Recently, Kim & Rolfe (1988) had similar observations, even after an initial separation of immunoglobulins in a sephadex column, from the colostrum and milk of hamsters after parenteral immunization with toxins A and B of *Clostridium difficile*. In both these studies and our own study, serum-derived IgG antibodies present in colostrum and milk of immunized animals prevented colonization and/or disease.

Sensitivity of *C. jejuni* to the complement-mediated bactericidal activity of vaccinated mouse serum and milk has been demonstrated in these experiments. There was a good correlation, in some instances, between the homologous bactericidal activity *in vitro* and homologous protection *in vivo*, including that induced by aflagellate live and heated vaccines given intraperitoneally. All vaccines given intraperitoneally stimulated a good specific IgG response and a good bactericidal activity, including strain 20186 vaccine which gave limited protection, and boiled 81116 vaccine which did not protect at all. So, although a correlation could be drawn between specific IgG levels and bactericidal activity, no such relationship could be seen between the latter and protection. Some vaccinated dams with high serum and milk bactericidal titres did not protect their young *in vivo* e.g. heated (62 °C) strain 20186 and boiled strain 81116 vaccines.

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It has been shown elsewhere (Abimiku & Dolby, 1988) that there is limited cross-protection by strains sharing the LIO serotype antigens of the vaccine strain. The cross-protection in mice vaccinated with 81116 (PEN 6 LIO 6) ranged from 43% for strain 13024, PEN 29 LIO 6 to 65% for strain 12066, PEN 25 LIO 6 challenges. There was no cross-protection against strains which shared the PEN serotype antigen or which were completely heterologous to the vaccine strain. The serum and milk from that study was used here in bactericidal test. The findings indicate that it is unlikely that bactericidal activity plays any role in cross-protection, as only one LIO matched strain was killed *in vitro* by sera and milk shown to have bactericidal activity against homologous strains. The only strain to be killed, strain 12066, showed many similarities to the vaccine strain (81116) when examined by Western blotting and immunogold labelling (Abimiku & Davies, unpublished).

Infants were not protected by vaccinated dams against strains with matched PEN serotype antigens and there was no bactericidal activity *in vitro* against these strains in the serum or milk of vaccinated dams. The PEN serotyping scheme is based on the lipopolysaccharide (LPS) of *C. jejuni*, and LPS is known to bind to antibody to activate both alternative and classical complement pathways (Morrison & Kline, 1977). Furthermore, anti-LPS has been implicated as bactericidal antibody for Enterobacteraceae species and other organisms. The bactericidal antibody elicited by boiled *C. jejuni* vaccine seems to suggest that heat-stable antigen such as LPS might be involved. However, matched PEN strains were not susceptible to serum bactericidal activity. This indicates either that LPS of matched PEN strains may not be as similar as the typing scheme seems to suggest, or else the antigen stimulating bactericidal activity is something as well as or other than the LPS.

From our experiments, therefore, we have some limited correlations of *in vitro* bactericidal activity of mouse serum and milk with protection of infant mice against gut colonization by *C. jejuni*. We have equally clear demonstrations that infant mice are not always protected by dams with high bactericidal titres to the challenge strain, and that bactericidal titres do not correlate well with the degree of cross-protection. However, it is interesting that high IgG antibody response always correlated with a good bactericidal titre. Unlike IgA, this class of antibody, along with IgM, are good activators of the classical complement pathway (Taylor, 1983). Our findings are in keeping with those with other pathogens. For example, in *Bordetella pertussis* infection (also of a mucosal surface) bactericidal activity and protection do not correlate (Ackers & Dolby, 1972), and in meningococcal infection, a serum bactericidal response may not be as important as previously considered (Blakebrough *et al.* 1983; Hassan-King *et al.* 1988). Narendranathan and co-workers (1988) recently showed that for *Vibrio cholerae*, the levels of antibody (mainly anti-LPS) present did not always account for the immune status established in rats. The situation has been complicated even further by the presence of, and possible role played by, serotype-specific vibriocidal antibodies stimulated by proteins and not LPS. The situation may be as complicated for *C. jejuni* infections where the protection achieved *in vivo* could be the sum total of a number of antibody systems operating simultaneously. Therefore, a single serological test may be inadequate to account for the presence or role of these antibodies.

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