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Monoclonal antibodies directed against the flagella of *Campylobacter jejuni*: production, characterization and lack of effect on the colonization of infant mice

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SUMMARY

Eight monoclonal antibodies have been derived from Balb/c mice hyperimmunized with the purified flagella from *Campylobacter jejuni* strain 81116. These monoclonal antibodies are directed against flagella as demonstrated by reaction in ELISA against flagellate and aflagellate antigens, radio-immunoprecipitation and electro-immunoblotting techniques. Some of the antibodies react with a 60K minor protein as well as the 62K flagella protein. This protein may be related to an antigen expressed on the surface of the organism and detectable by immunogold labelling with one of the monoclonal antibodies.

None of the antibodies causes the aggregation of bacteria or inhibits bacterial motility, unlike polyclonal anti-flagella antiserum. Moreover, none of the antibodies tested protected infant mice from colonization with *C. jejuni* strain 81116 even though partial protection (28%) was observed with syngeneic anti-flagella antiserum. Absence of protection is probably due to the cryptic nature of the flagella epitopes investigated or lack of antibody activity in the gastrointestinal tract.

INTRODUCTION

Campylobacter jejuni is now recognized as a major cause of acute bacterial enteritis in the UK. The outer membrane proteins and surface antigens of *C. jejuni* have been described (Logan & Trust, 1982) and flagella identified as comprising a major diffuse 62K protein and a minor 84K protein by comparison with an aflagellate variant (Newell, McBride & Pearson, 1984). The flagella of *C. jejuni* are highly antigenic inducing the production of cross-reacting antibodies during naturally-acquired human infections (Newell, 1985; Wendow *et al.* 1985; Nachamkin & Hart, 1985). Experimental evidence suggests that flagella have an important role in the pathogenesis of campylobacter infections. Aflagellate variants and mutants poorly colonize the gut of infant mice (Newell, McBride & Dolby, 1985; Morooka, Umeda & Amako, 1985) and man (Skirrow, 1983). Moreover, flagella appear to have adhesive properties distinguishable in *in vitro* attachment models using aflagellate and/or non-motile variants (Newell, McBride & Dolby, 1985). Flagella, therefore, may be a potential candidate component for a vaccine against campylobacterosis.

Animal models to investigate the pathogenic mechanisms of campylobacter infection have been difficult to establish (Newell, 1984). Infected infant mice rarely demonstrate symptoms of disease but are consistently colonized by *C. jejuni* (Field *et al.* 1981, Newell & Pearson, 1984). Recent investigations indicate that infant mice can be immunologically protected from colonization by vaccination of the dams (Dolby & Newell, 1985).

This report describes attempts to passively immunize infant mice with monoclonal antibodies directed against flagella. The specificity of the monoclonal antibodies investigated have been demonstrated by ELISA, radio-immunoprecipitation and electro-immunoblotting. Some of the flagella antigens have been localized by immunogold ultrastructural labelling techniques. None of the monoclonal antibodies protected infant mice from colonization with *C. jejuni*, although partial protection was observed with mouse anti-flagella antiserum.

MATERIALS AND METHODS

Bacterial strains. C. jejuni strain 81116 and the aflagellate variant isolated from this strain (SF-2) have been previously described (Newell, McBride & Pearson, 1984).

Antigen preparation. Outer membrane proteins and purified flagella were prepared from *C. jejuni* 81116 as previously described (Newell, McBride & Pearson, 1984).

Monoclonal antibody production. Adult female Balb/c mice were immunized with 10 μg outer membrane proteins in Freund's complete adjuvant by multiple subcutaneous injections. The mice were boosted twice, at 3-weekly intervals with 10 μg of flagella in Freund's incomplete adjuvant at multiple subcutaneous sites then finally with 20 μg flagella intravenously 3 days prior to harvesting the spleen. Immune spleen cells were hybridized with NS-1 mouse myeloma cells (Flow Labs Ltd, originally obtained from Dr C. Milstein) using the technique of De St Groth & Scheidegger (1980). Supernatants were screened for anti-campylobacter activity by the ELISA technique. Monoclonal antibodies with anti-flagella activity were selected by ELISA using sonicates of strains 81116 and SF-2 as antigens. Antibody secreting cells were cloned twice.

Ascitic fluid production. Adult Balb/c mice were injected with 0·5 ml of Pristane (Sigma Chemical Co) intraperitoneally, 7 days later the mice were injected intraperitoneally with 1×10^7 hybridoma cells in 0·5 ml of PBS and the ascitic fluid produced collected.

ELISA technique. Antigen (10 μg/ml whole cell sonicate, 1 μg/ml flagella or 5 μg/ml outer membrane preparation) in 100 μl of 0·1 M carbonate buffer, pH 9·6, was absorbed onto microELISA plates (Dynatech Ltd) overnight at 20 °C. The wells were washed in ELISA wash (0·85 % (w/v) sodium chloride containing 0·05 % (v/v) Tween 20) and incubated with appropriate dilutions or ascitic fluid for 2 h at 37 °C. After washing the wells were incubated with 100 μl of rabbit anti-mouse IgG coupled to peroxidase (Miles Research Labs) (1:1000 dilution in ELISA wash containing 1 % (w/v) bovine serum albumin and 0·6 % (w/v) TRIS, pH 7·6) for 2 h at 37 °C. Bound antibody was detected with 100 μl of tetra-methyl-benzidine substrate for 15 min, stopped with 50 μl 2 N sulphuric acid and read at 450 nm in a microELISA reader (Dynatect Ltd).

Determination of antibody isotype. Antibody isotype was determined by micro-ELISA. After incubation of the supernatant with antigen coated wells the bound antibody was incubated with 1:1000 dilution of rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (Litton Bionetics Inc, Kensington M.D.) bound rabbit antibody was detected with 1:1000 dilution of goat anti-rabbit IgG coupled to peroxidase (Miles Research Labs).

Radio-immunoprecipitation. Flagella preparations were radiolabelled with methyl 3,5-di[^{125}I]iodohydroxybenzimidate as previously described (Newell, McBride & Pearson, 1984) and solubilized in 3% (v/v) Empigen BB, 0·1% (w/v) SDS in PBS. Insoluble material was removed by centrifugation at 100000 ***g*** for 1·5 h, the ^{125}I-labelled flagella were incubated with supernatant and precipitated with Sepharose 4B coupled to rabbit anti-mouse IgG (Sigma Chemicals Ltd).

SDS–PAGE. A 10–25% (w/v) linear gradient SDS–PAGE system was used (Lambden *et al.* 1979). Protein bands were stained with Kenacid Blue R (BDH, Chemicals) The protein molecular weight markers were trypsin, ovalbumin, bovine serum albumin and lysozyme.

Electro-immunoblotting. SDS–PAGE gels of *C. jejuni* strains 81116 and SF-2 and purified flagella were electro-blotted onto nitrocellulose (Newell, McBride & Pearson, 1984). Non-specific protein binding was eliminated by incubation with bovine serum albumin. The nitrocellulose was then incubated with 1:2 dilution of supernatant. Bound antibody was detected with ^{125}I-labelled sheep anti-mouse IgG (Amersham International), specific activity 5–20 μCi μg^{-1}.

Immunogold labelling. Bacteria were suspended in 0·1% (v/v) glutaraldehyde in 0·1 M phosphate buffer, pH 7·2 for 15 min at 20 °C then washed by centrifugation (3000 ***g***, 15 min) and resuspended in 0·1 M phosphate buffer, pH 7·2 and stored at 4 °C until required. This pre-fixed bacterial suspension was centrifuged for 4 min in a microcentrifuge and the cell pellet resuspended in 10 μl of diluted ascitic fluid, mouse anti-flagella antisera or normal mouse sera (1:100 dilution in PBS) for 2 h at 37 °C. After incubation the cells were washed three times in PBS and resuspended in 20 μl of PBS. One drop of this cell suspension was placed on a formvar-carbon-coated grid. Excess fluid was removed by blotting paper. The grid was then inverted onto a 10 μl droplet of gold reagent (Hodges, Smolera & Livingston, 1984) (goat anti-mouse IgG coupled to 15 nm gold particles; kindly supplied by Dr G. Hodges, I.R.C.F., London) on Gelbond film (Miles Research Labs Ltd) and incubated at 4 °C overnight. The grids were washed three times on droplets of PBS, negatively stained with 0·25% (w/v) phosphotungstic acid and viewed on a Philips 201 transmission electron microscope at 60 kV.

Aggregation and inhibition of motility. C. jejuni strain 81116 was grown overnight on blood agar plates and one loopful of bacteria was suspended in PBS at 20 °C. Drops of this cell suspension were placed on multiwell slides and mixed with an equal volume of diluted mouse anti-flagella antiserum, normal mouse serum or ascitic fluid. Motility and aggregation were observed by dark ground microscopy on a Leitz Dialux Microscope fitted with a dark ground condenser and 100X oil immersion dark ground objective. Aggregation and motility were assessed subjectively as strongly positive (+ + +) to negative (−).

Protection and challenge of infant mice. Four-day-old infant Balb/c mice were injected intraperitoneally with 50 μl of either ascitic fluid, syngeneic mouse anti-flagella antiserum or PBS. Twenty four hours later the mice were intragastric-

ally challenged with approximately 1×10^7 organisms of *C. jejuni* strain 81116 in 50 μl of PBS as previously described (Newell, McBride & Dolby, 1985). The extent of colonization in the caecum and colon was determined from viable counts of the homogenized material at 6 days post-infection. This time period was adopted because by 6 days post-infection avirulent campylobacters are largely eliminated from the gastrointestinal tract (Newell, McBride & Dolby, 1985). At least six mice were used in each group and each experiment was repeated at least once. The degree of protection was assessed as previously described (Dolby & Newell, 1985). Briefly infants who were fully protected (colonization 10000 times less than the controls or no organisms isolated) were given a numerical value of 1. Infants who were partially protected (colonization 100–10000 times less than the controls) were given a value of 0·5 whilst infants who were unprotected were given a value of 0.

RESULTS

Characterization of the monoclonal antibodies

The properties of the eight monoclonal antibodies investigated are shown in Table 1. Each monoclonal antibody reacted with sonicated strain 81116 and purified flagella in ELISA. Only CF1 and CF2 reacted to any extent with the aflagellate strain, SF-2. Each antibody precipitated a single 62K protein from ^{125}I-labelled flagella (Fig. 1). This protein was not precipitated by two control monoclonal antibodies. However, two proteins, a major 62K protein and a minor 60K protein, were labelled by five of the antibodies in immunoblots of SDS–PAGE gels of total protein profiles of strain 81116 (Fig. 2) while CF3, CF4 and CF13 only labelled the higher molecular weight protein band.

Immunogold localization of flagella antigens

Using an indirect immunogold labelling technique at the ultrastructural level polyclonal mouse anti-flagella antiserum randomly labelled the shafts of the flagella (Fig. 3). Normal mouse serum gave no specific labelling of the bacteria. The monoclonal antibodies CF3, CF4 and CF13 specifically labelled the tips and broken pieces of the flagella (Fig. 4). The monoclonal antibody CF1 randomly labelled the bacterial surface of strain 81116 and the aflagellate variant, SF-2, but did not label the flagella (Fig. 5). No specific flagella or surface labelling was observed with the monoclonal antibodies CF2, CF5, CF6 and CF7.

Inhibition of motility and bacterial aggregation

None of the monoclonal antibodies caused any detectable aggregation or reduction in motility of bacteria (strain 81116) at any dilution tested. Polyclonal anti-flagella antisera caused a significant reduction in motility until a dilution of 1:2560 when motility became comparable with normal mouse serum. Similarly anti-flagella antiserum caused a significant aggregation of bacteria which was reduced to that of the normal mouse serum at a dilution of 1:1280. The combination of monoclonal antibodies appeared to have no effect on the lack of inhibition of motility or aggregation.

Table 1. *Monoclonal antibodies: isotypes and ELISA titres of ascitic fluid against flagellate and aflagellate antigens*

Clone	Ig subclass	ELISA titre* × 100		
		81116	SF-2	Flagella
CFl	G2a	1100	4·8	18
CF2	G2a	10700	1·2	21000
CF3	G2a	14800	0·2	8200
CF4	G2a	28300	0·3	78100
CF5	M	53800	0·4	5900
CF6	G3	500	0·3	800
CF7	M	5600	0·3	1600
CF13	G2b	4800	0·3	18300

* Obtained by the extrapolation of the linear portion of antibody dilution vs OD_{450} curve to $OD_{450} = 0·1$.

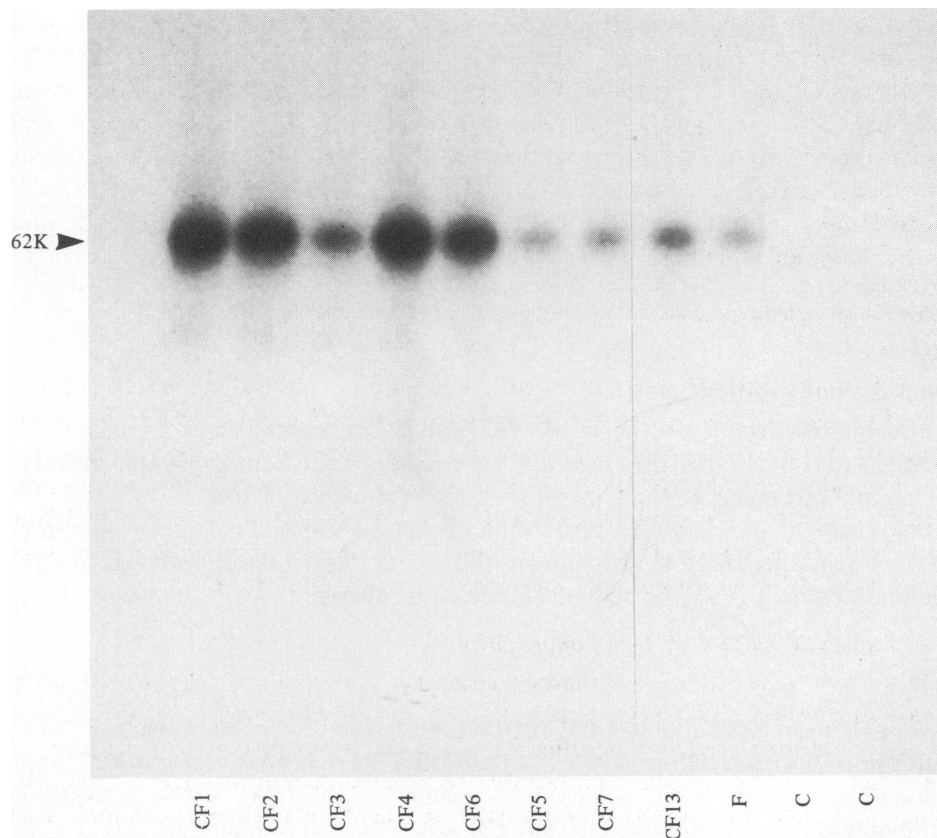

Fig. 1. Radio immunoprecipitation of ^{125}I-labelled flagella by monoclonal antibodies. The controls were monoclonal antibodies directed against *C. jejuni* antigens other than flagella. Track F is ^{125}I-labelled flagella.

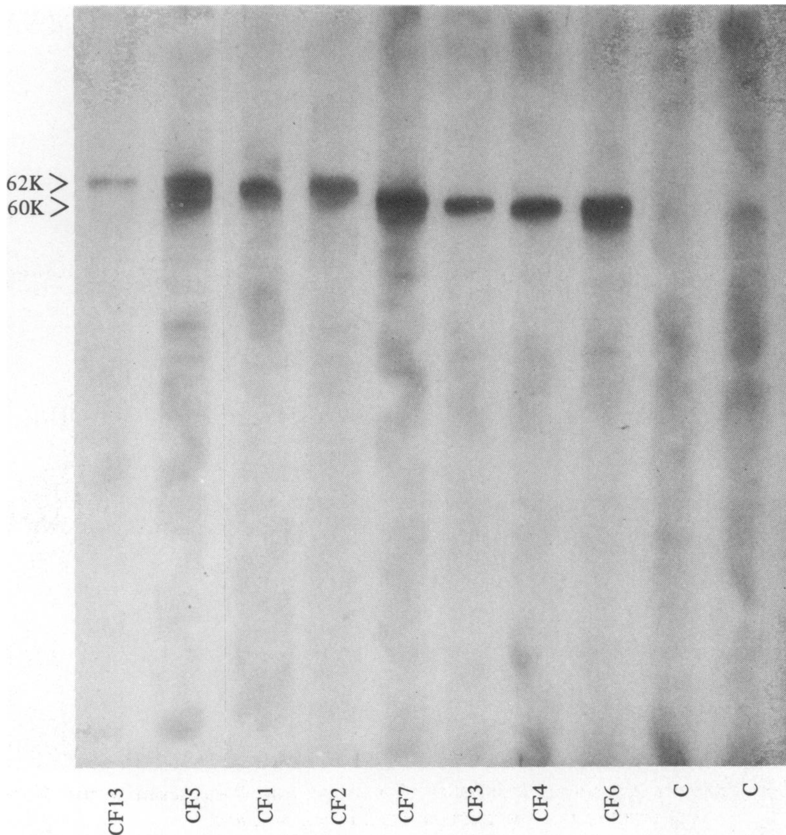

Fig. 2. Electro immunoblot of whole protein profiles of *C. jejuni* strain 81116 incubated with anti-flagella monoclonal antibodies. The controls are the same as Fig. 1.

Passive protection of infant mice

The anti-flagella activity of the ascitic fluid and the antiserum given to the infant mice and the activity in the infant mouse serum and in the homogenized contents of the caecum and colon at the time of sacrifice is shown in Table 2.

The colonization and degree of protection of the caecum and colon of the infant mice 6 days post-challenge is also shown in Table 2. None of the infected infant mice demonstrated any symptoms of diarrhoea or disease.

DISCUSSION

The properties of eight monoclonal antibodies, derived from the spleens of mice hyperimmunized with *C. jejuni* strain 81116 flagella, have been investigated. These monoclonal antibodies were selected to react, in ELISA, with flagellated campylobacters and purified flagella but not aflagellate organisms. In RIPA and immunoblotting the antibodies react with a 62K protein which has the same properties as the 62K flagella protein. The characteristics of this panel of monoclonal antibodies, including differential reaction with serotype strains of *C. jejuni* or *C. coli* and competitive ELISA assays (unpublished data) indicate that there

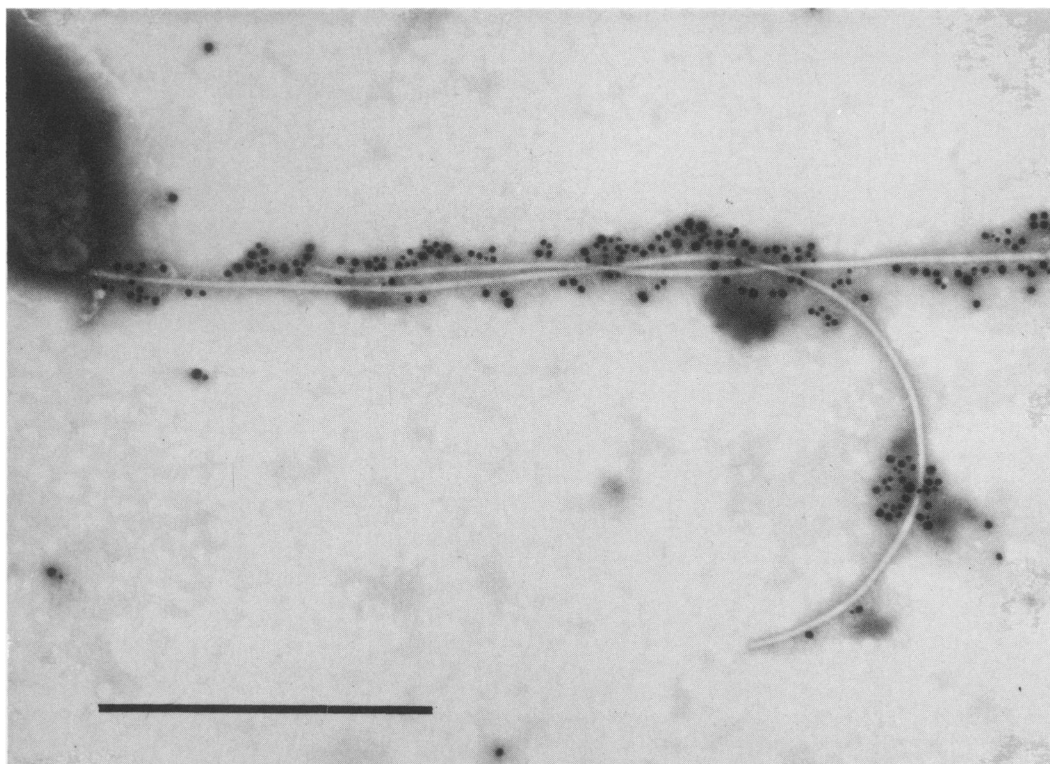

Fig. 3. *C. jejuni* flagella immuno-gold labelled with mouse anti-flagella antiserum. Note the labelling of the flagella shafts. Bar = 1 μm.

Table 2. *Anti-flagella antibody activity in treated mice and the degree of protection against colonization – representative experiment*

Clone	Average ELISA titre × 100			Degree of protection			% Protection
	Original	Serum†	Intestinal†	+	±	–	
CF1	180	154	< 20	0	0	6	0
CF2	215580	166470	< 20	0	0	7	0
CF3	781250	10711	< 20	0	0	8	0
CF4	82070	20122	< 20	0	0	8	0
CF6	8620	933	< 20	0	0	6	0
CF13	18353	4304	< 20	0	0	8	0
Antiserum	89100	250	< 20	0	4	3	28
N.m.s.	< 20	< 20	< 20	0	0	9	0

* Serum or ascitic fluid.
† At 6 days post-infection.

are at least six epitopes distinguishable on the flagella protein. Although all the monoclonal antibodies appear to be directed against the flagella protein of *C. jejuni* some react with a minor 60K protein, immunogold label a surface antigen and partially react with the aflagellate variant in ELISA. These results may be explained by the presence of other flagella-like antigens expressed on the surface

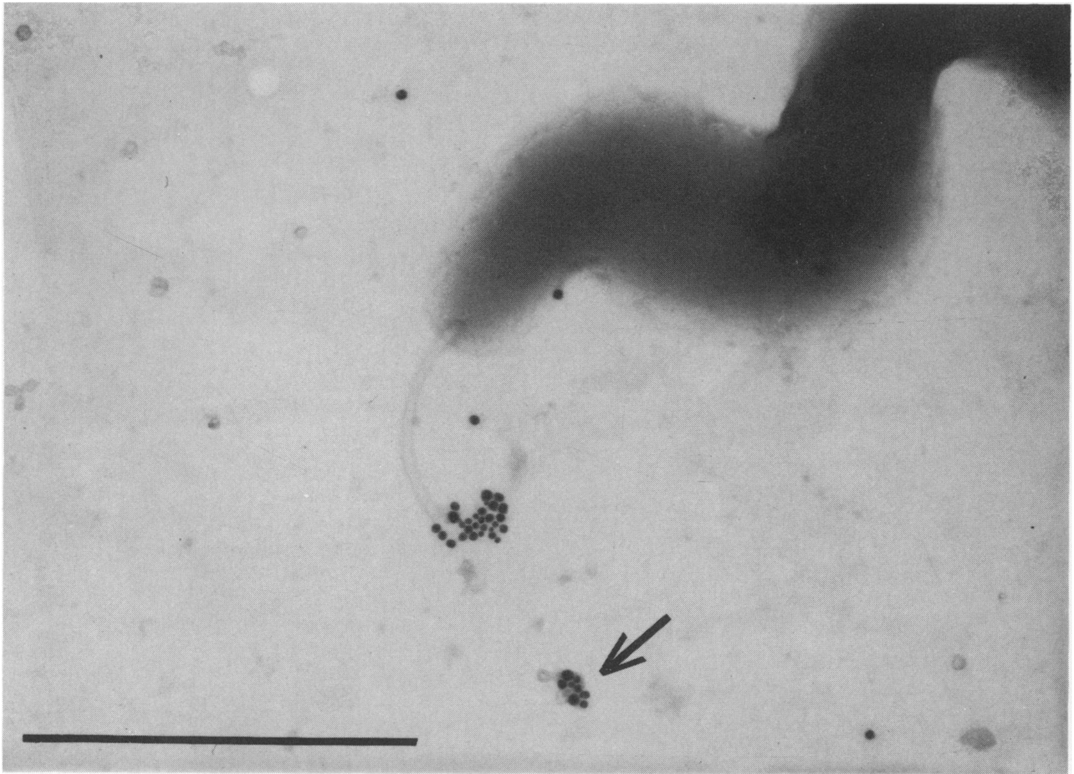

Fig. 4. *C. jejuni* flagella immunogold labelled with monoclonal antibody CF13. Note the labelling of the tip of the flagellum and a broken piece of flagellum (↑). Bar = 1 μm.

of the organism. The expression of multiple flagella antigens, with varying molecular weights, by *C. jejuni* is supported by recent immunoblotting studies with matching human convalescent sera and clinical strains (Newell, 1985). Moreover, flagella antigens may be detected, using immunoblotting techniques, in the aflagellate variant by rabbit anti-flagella sera (Newell, McBride & Pearson, 1984). The relationships between the 62K and the 60K proteins and the surface antigens detected by CFl and rabbit anti-flagella antisera are under investigation.

Immunogold labelling with anti-flagella antisera have been used to visualize antigens along the flagella shaft, but none of the monoclonal antibodies localized these antigens. However, some antibodies specifically labelled the tips and one end of broken pieces of flagella, suggesting that the antigens were only exposed at these sites. Inadequate exposure of these flagella antigens could explain the absence of agglutination and inhibition of motility by the monoclonal antibodies but not by the anti-flagella antiserum.

None of the antibodies, when administered intraperitoneally protected infant mice from colonization with *C. jejuni* strain 81116. Nevertheless, infant mice were partially protected with syngeneic anti-flagella antiserum. Although the purity of the flagella antigen used to obtain this antiserum has been previously established (Newell, McBride & Pearson, 1984) the specificity of the antiserum cannot be guaranteed. There are several possible explanations for the absence of passive

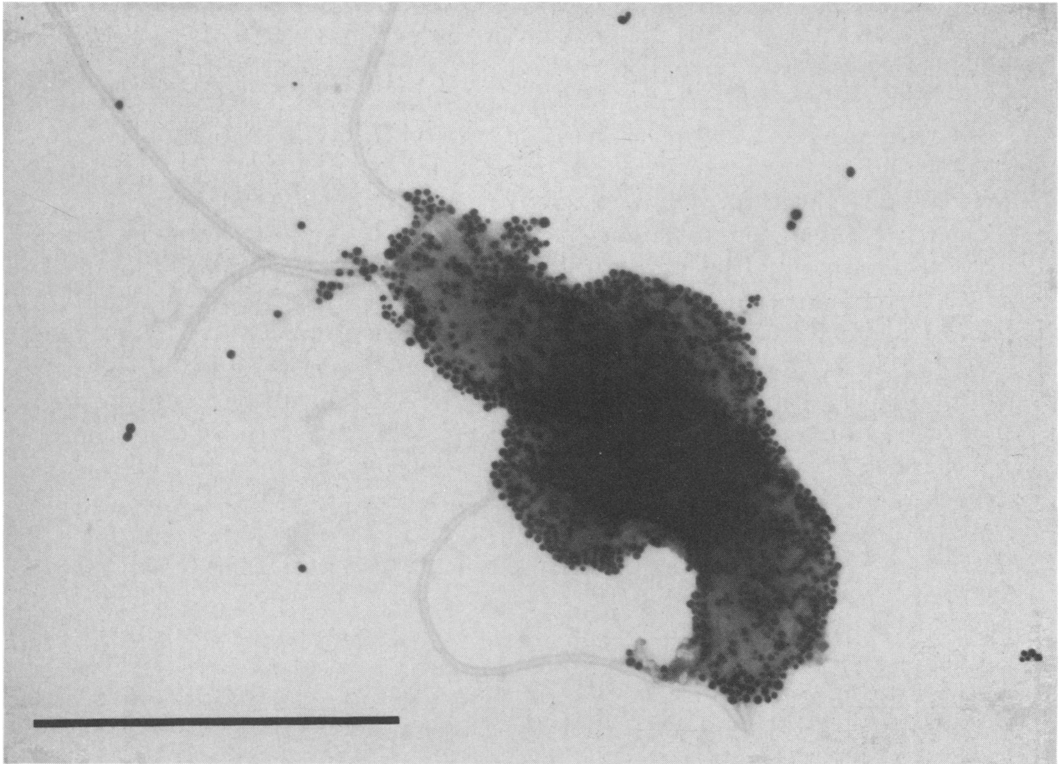

Fig. 5. *C. jejuni* immunogold labelled with monoclonal antibody CF1. Note the labelling of the bacterial surface but not the flagella. Bar = 1 μm.

protection by the monoclonal antibodies. Firstly, the poor antibody titres in the caecal and colonic contents suggests that the antibodies are unable to traverse the intestinal mucosa from the circulation and penetrate and survive in the intestinal lumen. The monoclonal antibodies of IgM subclass were not used in the protection studies because it was unlikely that they would be transported across the gastrointestinal mucosa. No attempt was made to administer the antibodies intragastrically before or at the same time as the challenge as the relevance of such a model to natural infection would be difficult to assess. The negligible titre of anti-flagella activity in the intestinal tissue of mice treated with antiserum suggests that lack of detectable antibody activity rather than penetration is the major problem. Antibody detection could have been limited by destruction of antibody activity either as a result of the method of tissue preparation or by protease activity within the homogenized material. Secondly the immunogold labelling and absence of aggregation and inhibition of motility indicate an inaccessibility of the flagella antigens to these antibodies. It seems most likely that protective antibodies would be directed against epitopes exposed along the length of the flagella shaft. However, the production of monoclonal antibodies against these antigens has so far proved to be difficult, even though such activity is present in the anti-flagella antiserum of the hyper-immunized mice. Thirdly, despite the highly antigenic nature of the flagella during campylobacter infections the anti-flagella antibody

response may not be protective. Recent studies using dams immunized with *C. jejuni* strains 81116 or SF-2 show that circulating maternal anti-flagella antibodies did not confer additional protection on infant mice (Dolby & Newell, 1985). However, fostering experiments suggest that, in this model, humoral immunity plays a minor role compared with mucosal immunity (Hassan & Dolby, 1985) which makes the infant mouse model a very difficult one in which to assess antibody-mediated protection, though unfortunately it is the only one available.

Despite the absence of protection of infant mice by the anti-flagella monoclonal antibodies described in this report it is still possible that the significant immune response induced by campylobacter flagella during natural infection could be exploited in the development of a vaccine and this is supported by the partial protection observed with syngeneic anti-flagella antisera. Nevertheless, at least some of the flagella epitopes are not protective in the infant mouse model. Moreover, some of our earlier studies (Dolby & Newell, 1985) indicate that flagella is not the only antigen relevant in protection and suggest that more investigations in this field are justified.

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