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Monoclonal antibodies directed against the flagella of *Campylobacter jejuni*: cross-reacting and serotypic specificity and potential use in diagnosis

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SUMMARY

Nine monoclonal antibodies directed against the flagella of *Campylobacter jejuni* strain 81116 have been investigated for serotypic and cross-reacting activity using a panel of 17 Penner serotype strains of *C. jejuni*. Four monoclonal antibodies were exclusively specific for serotype-6 strains, which was the serotype of *C. jejuni* strain 81116. Two monoclonal antibodies cross-reacted with all the flagellated strains of *C. jejuni* tested. One of these cross-reacting monoclonal antibodies, CF5, was found to react with all other *Campylobacter* species except *C. sputorum bulbulus* but it did not react with other bacterial enteropathogens. An antigen-capture ELISA technique was established, using this monoclonal antibody, which could detect flagellar antigen in human faecal material. These anti-flagella monoclonal antibodies therefore may be valuable in the diagnosis and serotyping of *C. jejuni* in clinical material.

INTRODUCTION

Campylobacter jejuni is now recognized as a major cause of human acute bacterial enteritis throughout the world. Although the disease is generally self-limiting, early antibiotic treatment may alleviate the discomfort and pain and reduce the excretion period (Mandal & Ellis, 1983). Unfortunately, the isolation of *C. jejuni* from faeces requires a 48 h incubation time, in the presence of selective antibiotics, under demanding microaerophilic conditions. Such a diagnostic procedure is not conducive to early treatment.

The flagella of *C. jejuni* have been shown to be an important component of the bacterial surface, mediating motility and possibly adherence to intestinal epithelium (Newell, McBride & Dolby, 1985; Morooka, Umeda & Amako, 1985) as well as inducing a major antibody response during human disease (Nachamken & Hart, 1985; Newell, 1985; Wenman *et al.* 1985). A number of monoclonal antibodies have been produced directed against the flagella of *C. jejuni* in order to investigate their protective capacity during the colonization of infant mice (Newell, 1986). These monoclonal antibodies define at least six epitopes on the flagella protein. In this report the cross-reactivity of these monoclonal antibodies against a panel of *C. jejuni* strains of different serotypes has been determined. The results indicate that monoclonal antibodies directed against flagella may be used to detect

cross-reactive as well as serotypic antigens. The potential of one of the cross-reacting antibodies to detect antigen in human faecal material has also been investigated.

MATERIALS AND METHODS

Bacterial strains

The source and properties of *C. jejuni* strain 81116 and its aflagellate variant (SF-2) have been previously described (Newell, McBride & Pearson, 1984). The Penner serotype strains 1–18 (excluding strain 4) were provided by Dr J. Penner (Toronto, Canada) and serotyped in the Lior serotyping scheme by Dr. D. Jones (P.H.L.S., Manchester).

The type strains of *Campylobacter* species were obtained from the National Collection of Type Cultures (Colindale, London) and Dr M. B Skirrow (Worcester). All other clinical strains of *C. jejuni* were provided by Dr. A. D. Pearson (P.H.L.S., Southampton) and serotyped by Dr. J. Penner and Mr H. Lior (Ottawa, Canada). Other bacterial strains were provided by the PHLS Laboratory Southampton and the Division of Enteric Pathogens, CPHL, Colindale.

All campylobacter strains were cultured as previously described (Newell, McBride & Pearson, 1984).

Monoclonal antibodies

The production and characterization of the monoclonal antibodies has been previously described (Newell, 1986). Briefly, adult Balb/c mice were primed with 10 μg of sarkosyl insoluble outer membrane preparation (Newell, McBride & Pearson, 1983) from *C. jejuni* (strain 81116). Subsequently the mice were boosted with flagella (10 μg) purified from *C. jejuni* (strain 81116). Immune spleen cells were hybridized with P3-NS1/1-Ag4-1 (Flow Laboratories, originally from Dr Milstein) at a ratio of 1:1 using the polyethylene glycol technique as described by De St Groth & Scheidegger (1980).

Anti-flagella antibody secreting hybridomas were selected from those clones giving a positive reaction with the antigen sonicate from the flagellated wild type (*C. jejuni* strain 81116) but giving a negative or poor reaction with the antigen sonicate from the aflagellate variant (SF-2). The specificity of the monoclonal antibodies against flagella was confirmed by ELISA against purified flagella, radioimmunoprecipitation of ^{125}I-labelled flagella and immunoelectroblotting techniques (Newell, 1986).

ELISA technique

Ten μg/ml of bacterial sonicate or 1 μg/ml purified flagella in coupling buffer (0·05 M carbonate buffer, pH 9·6) were incubated in microELISA plates (Dynatech Labs., Billingshurst) (100 μl) overnight at room temperature. The wells were washed thoroughly in ELISA wash (0·85% saline with 0·05% Tween 20), then incubated with 100 μl of culture supernatant (1:2 dilution in ELISA wash) for 2 h at 37 °C. After washing the wells were incubated with 100 μl of rabbit anti-mouse IgG coupled to peroxidase (1:1000 dilution in ELISA wash containing 1% BSA and 0·5% Tris, pH 7·6) (Miles Research Labs.) for 2 h at 37 °C. The wells were washed and then incubated with 100 μl of substrate (10 mg *O*-phenylene diamine

dissolved in 1 ml warm methanol and diluted to 100 μl with water containing 10 μl hydrogen peroxide) for 1 h at 37 °C in the dark. The reaction was stopped with 50 μl of 2 M sulphuric acid and the optical density read at 490 nm in a microELISA reader (Dynatech Labs).

Antigen capture ELISA technique

Rabbits were immunized with 20 μg of sarcosyl insoluble outer membrane from strain 81116 (Newell, McBride & Pearson, 1984) subcutaneously and intramuscularly in Freund's complete adjuvant. The rabbits were boosted four times at approximately fortnightly intervals by multiple subcutaneous injections with the same antigen dose in Freund's incomplete adjuvant. An IgG fraction of rabbit anti-outer membrane preparation was prepared by ammonium sulphate precipitation and dialysed against phosphate-buffered saline overnight. This IgG fraction was coupled to ELISA plates at a concentration of 10 μg/ml in coupling buffer overnight at 20 °C then washed with ELISA wash. Sonicates of *C. jejuni*, purified flagella or faecal material (100 μl) were added to the wells and incubated for 2 h at 37 °C. After washing the wells were incubated with culture supernatant (1:2 dilution) or ascitic fluid (1:1000 dilution) for 2 h at 37 °C. Bound mouse antibody was detected as described above.

Immunodot technique

The reactivity of the monoclonal antibodies with whole bacteria was also monitored by an immunodot technique modified from Hawkes, Niday & Gordon (1982). Bacterial suspensions (10 μl of 10 μg/ml) in phosphate-buffered saline were dotted on to nitrocellulose paper and incubated at 4 °C for 10 min. The paper was washed in 50 mM TRIS, 200 mM sodium chloride, pH 7·4 (TBS), for 10 min then the endogenous peroxidase was blocked with 1·6% hydrogen peroxide in methanol. After a brief wash the non-specific binding was blocked with blocking buffer (3% BSA in 10 mM Tris and 0·9% sodium chloride, pH 7·4) for 2 h at 37 °C and the nitrocellulose paper was incubated with undiluted culture supernatant overnight at 4 °C or for 2 h at 37 °C. The nitrocellulose paper was then washed in several changes of TBS before incubation in rabbit anti-mouse IgG conjugated to peroxidase (1:1000 dilution in blocking buffer) (Miles Research Laboratories) for 2 h at 37 °C. After washing extensively in TBS the nitrocellulose paper was incubated in the substrate (1 ml 4-chloro 1 naphthol (Koch–Light Laboratories Ltd) dissolved in methanol (3 mg/ml) diluted to 6 ml with TBS plus 2 μl of hydrogen peroxide).

RESULTS

The cross-reactivity of the monoclonal anti-flagella antibodies with the Penner serotype strains (and the corresponding Lior serotypes) as determined by ELISA is shown in Table 1. On the basis of Penner serotype specificity four groups could be distinguished: group 1, including CF1, CF2, CF13 and CF14, which reacted with Penner serotype 6 strain exclusively; group 2, including CF3 and CF4, which reacted with Penner serotypes 6 and 11; group 3, including CF6, which reacted with a number of serotypes; and group 4, including CF5 and CF7, which reacted with all Penner serotypes except 8, 15 and 16 and reacted poorly with strain 13.

Table 1. *Cross-reactivity of monoclonal antibodies with Penner serotype strains*

		Penner serotype strains																
	Ig	1	2	3	5	6	7	8*	9	10	11	12	13*	14	15*	16*	17	18
		Lior serotype																
Clone	Class	2	4	1·2	9	6	NT	NT	1·2	9	8	2	7	4	NT	7	NT	11
CF1 CF13 CF2 CF14	G2a G2b G2a G2a	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
CF3 CF4	G2a G2a	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−
CF6	G3	+	−	+	+	+	+	−	+	−	−	+	−	+	−	−	−	+
CF5 CF7	M M	+	+	+	+	+	+	−	+	+	+	+	−	+	−	−	+	+

* Aflagellate or poorly flagellate.
† As determined by Dr D. Jones, P.H.L.S., Manchester.

Table 2. *Reactivity of monoclonal antibodies CF1 and CF2 with* Campylobacter jejuni *strains of Penner serotype 6*

		Serotype		ELISA titre	
Strain	Source*	Penner	Lior	CF1	CF2
81116	S	6	6	1400	5400
Penner 6	P	6	6	1200	2000
66222	S	6	NT	700	3900
52872	S	6	NT	2800	8700
90746	S	6	6	1900	40
87372	S	6	NT	2000	3325
89181	S	6	6	1900	4500
75345	S	6	NT	1700	2400

* S, Dr A. D. Pearson, PHLS, Southampton; P, Dr J. Penner, Canada; NT, not tested.

When strains 8, 13, 15 and 16 were observed by electron microscopy they were found to be aflagellate or poorly flagellated.

Monoclonal antibodies of group 1, which were specific for the serotype 6 strain, were tested against six other *C. jejuni* strains known to be Penner serotype 6 but from unrelated sources (Table 2). The monoclonal antibodies reacted with all six strains tested.

The specificity of the cross-reacting antibody CF5 against other *Campylobacter* species (Table 3) and other bacterial enteropathogens (Table 4) was tested using both immunodot and ELISA techniques. All the campylobacters, except *C. sputorum bulbulus* reacted with CF5 in the immunodot technique. Neither *C. sputorum bulbulus* nor *C. laridis* reacted with CF5 in the ELISA technique. CF5 did not cross-react with any of the other bacterial enteropathogens tested.

An antigen-capture technique was established to detect *C. jejuni* flagella antigen. The reaction of the monoclonal antibody, CF5, with partially purified flagella in outer membrane preparations, is shown in Fig. 1. A 10% solution (in either phosphate-buffered saline or 0·2 M glycine-HCl buffer, pH 2·2) of human

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Table 3. *Reactivity of monoclonal antibody CF5 with* Campylobacter *species*

Species	Strain	Source	Immunodot	ELISA
C. jejuni	75345	S	+	+
	66222	S	+	+
	81116	S	+	+
	32777	S	+	+
	32534	S	+	+
	31914	S	+	+
	27906	S	+	+
C. coli	11353	NCTC	+	+
	11366	NCTC	+	+
	16059	W	+	+
C. laridis	3034	NCTC	+	−
	25428	W	+	−
C. fetus fetus	10842	NCTC	+	+
	25320	W	+	+
C. fetus veneralis	10354	NCTC	+	+
C. pyloridis	313	W	+	+
	314	W	+	+
C. sputorum bulbulus	11367	NCTC	−	−

NCTC, National Collection of Type Cultures; S, Dr A. D. Pearson, PHLS, Southampton; W, Dr M. B. Skirrow, Worcester.

Table 4. *Cross-reactivity of monoclonal antibody CF5 with non-campylobacter organisms*

Bacteria	No. of strains	Immunodot	ELISA
E. coli	3	−	−
Proteus	1	−	−
Pseudomonas	3	−	−
S. typhimurium	1	−	−
V. cholerae	1	−	−

diarrhoeic faeces (obtained from a patient with diarrhoea of confirmed viral origin and without a positive culture for *C. jejuni*) were mixed with *C. jejuni* flagella to give a final concentration of 1 μg of flagella/ml. In some cases the particulate material was removed by brief centrifugation (0·5 min at 12000 g) and the supernatant added to the rabbit anti-outer membrane IgG-coated wells in the antigen capture ELISA. Representative results of this experiment are given in Table 5.

DISCUSSION

A panel of nine monoclonal antibodies directed against the flagella of *C. jejuni* strain 81116 have been investigated for their potential usefulness in the diagnosis and serotyping of *C. jejuni* infections.

Several of the monoclonal antibodies had a broad specificity for the flagella of all the *C. jejuni* strains tested. Moreover the monoclonal antibody CF5 cross-reacted

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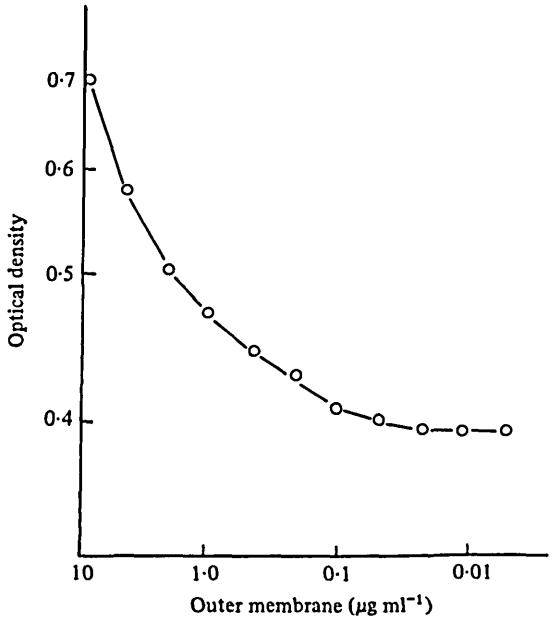

Fig. 1. The sensitivity of the antigen capture ELISA technique for flagella antigen in outer membrane preparations.

Table 5. *Detection of* C. jejuni *flagella in faecal material by antigen-capture ELISA*

	Average ELISA reading*	
	Diluent: ELISA wash	Diluent: glycine buffer
1 μg/ml flagella	487±7	915±17
10% faecal material	232±17	248±7
10% faecal material+1 μg/ml flagella	269±18	545±9
10% faecal material+1 μg flagella+centrifugation	241±7	650±16

* Average O.D. of four wells.

with most of the other *Campylobacter* species investigated. It is interesting to note that CF5 cross-reacts with gastric campylobacter-like organisms (*Campylobacter pyloridis*), particularly in view of doubts regarding the relationship between this organism and other campylobacters (Jones, Curry & Fox, 1985). CF5 did not react with other non-campylobacter bacterial enteropathogens tested. In order to consider the diagnostic potential of such an antibody an antigen-capture technique was established to detect campylobacter flagellar antigen. The solid phase comprised polyclonal rabbit anti-*C. jejuni* outer membrane. Sarkosyl insoluble outer membrane preparations of *C. jejuni* consist mainly of flagella and the major outer membrane protein (Newell, McBride & Pearson, 1984). The antigen capture technique successfully detected flagella in outer membrane preparations and sonicates of whole organisms. Moreover, preliminary experiments indicate that the antigen could be detected in faecal material to which flagella had been added,

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although solubilization of the antigen and removal of particulate material was first required. Further investigations are in progress to determine if this, or other immunological techniques, using this monoclonal antibody, may be useful in detecting *C. jejuni* in clinical material. Flagella appear to be an essential virulence factor for the successful colonization of infant mice (Newell, McBride & Dolby, 1985; Morooka, Umeda & Amako, 1985) and man (Skirrow, 1983). Aflagellate laboratory strains sometimes occur, presumably due to repeated subculture allowing the selection of non-motile variants (Newell, McBride & Dolby, 1985), but aflagellate clinical isolates are rare occurrences (Thomas, Chan & Ribeiro, 1980). Moreover, in diarrhoeic stools from patients with campylobacterosis, considerable quantities of flagella are observed in negatively stained preparations by transmission electron microscopy, even though organisms may be few and far between (unpublished observation). It would therefore seem that a technique which detected campylobacter flagella in clinical material would be a useful diagnostic tool.

Serotyping is a major tool in the investigation of outbreaks of campylobacter enteritis. Currently two serotyping schemes are in common use; the Penner serotyping scheme which uses an indirect passive haemagglutination technique to detect soluble heat-stable antigens, and the Lior scheme which uses a slide agglutination technique to detect heat-labile antigens. Both schemes have a number of advantages and disadvantages. Recent comparisons indicate that both schemes are valuable in the analysis of outbreaks (Kaijser & Sjogren, 1985; Patton, Barrett & Morris, 1985) but neither test is recommended in isolation for the surveillance of sporadic cases at present.

Several of the monoclonal antibodies investigated in this report have been shown to be specific for Penner serotype 6, which is a relatively uncommon serotype in large studies of human sporadic cases (Abbot *et al.* 1983; Kaijser & Sjogren, 1985; Patton, Barrett & Morris, 1985). The serotype of the immunizing strain is Penner 6; Lior 6 (Newell, McBride & Dolby, 1985). The Penner serotype specificity of these monoclonal antibodies is unexpected since flagella are not considered to have an important role in the Penner serotyping scheme. However, a number of the Penner serotype 6 strains used in this study are also Lior serotype 6 and flagellar antigen is known to be important in the Lior serotyping scheme (Wenman *et al.* 1985). Although Penner serotype 6 is a heterogeneous group comprising at least three Lior serotypes (Kaijser & Sjogren, 1985; Patton, Barrett & Morris, 1985) certain Lior and Penner serotypes appear to correlate closely (Patton, Barrett & Morris, 1985). These results suggest that, at least for Penner serotype 6 (and possibly Lior serotype 6), monoclonal antibodies directed against flagella can be serotype-specific and could therefore be of value in the serotyping of clinical isolates. Unlike the polyclonal antisera currently used in serotyping, such monoclonal antibodies, once established, may be produced in considerable quantities with predictable specificity. Moreover, these preliminary results indicate that monoclonal antibodies could be used to serotype campylobacters in clinical material without prior isolation, thus reducing the time required to detect outbreak sources.

On the basis of the reactivity of this panel of anti-campylobacter flagella monoclonal antibodies with Penner serotype strains, it is evident that the flagellar protein comprises multiple epitopes which may have serotypic or cross-reactive

specificity. This confirms previous preliminary studies using matching human convalescent sera and clinical isolates of *C. jejuni* (Newell, 1985). The relationships between these multiple antigens and other flagellar properties require further investigation, especially as flagella have been suggested as a vaccine candidate. However, the presence of cross-reacting antigens expressed on flagella further supports the potential use of flagella as a vaccine component.

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