Antigenic analysis of *Clostridium chauvoei* flagella with protective and non-protective monoclonal antibodies

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Five monoclonal antibodies (mAbs) directed against the flagellin of *Clostridium chauvoei* were used to analyse the structural and antigenic characteristics on the bacterial flagellar surface. Immune electron microscopy showed that three protective mAbs recognized the surfaced-exposed epitopes on the flagellar filament of this bacteria. In contrast, two non-protective mAbs recognized internal epitopes of the flagellar filament. These findings have been confirmed by ELISA using mAbs absorbed with whole cells of *C. chauvoei* possessing flagella. Competitive binding assays showed that protective mAbs indicated reciprocal competition, while each of the non-protective mAbs had topographically distinct epitopes. Moreover, immunoblotting analysis with cyanogen-bromide-cleaved flagellin showed that protective mAbs may preferentially recognize conformational epitopes, whilst one of the non-protective mAbs may recognize a linear and conformation-independent epitope in the flagellin of *C. chauvoei*.

Introduction

Many studies on the flagella of *Vibrio cholerae* (Attridge & Rowly, 1983), *Salmonella typhimurium* (Carsiotis *et al.*, 1984; Weinstein *et al.*, 1984), *Pseudomonas aeruginosa* (Montie *et al.*, 1982) and *Campylobacter jejuni* (Morooka *et al.*, 1985; Newell *et al.*, 1985) suggest that flagella may be important as a virulence factor in the pathogenesis of infections caused by these organisms. Further studies also suggest that flagella of *V. cholerae* (Yancey *et al.*, 1979; Resnick *et al.*, 1980) and *P. aeruginosa* (Montie *et al.*, 1987) may elicit production of protective antibodies against the respective infections.

Our previous studies have indicated that in *Clostridium chauvoei*, which causes blackleg in cattle, sheep and other ruminants, the flagella are important for protective immunity in mice. Purified flagella of *C. chauvoei* showed a protective effect, while the protective antigenicity of non-flagellated mutants was 100-fold less than that of the flagellated parent strain (Tamura *et al.*, 1984; Tamura & Tanaka, 1984). However, there are still many points to be clarified as to the nature of protective antigens on the flagella of *C. chauvoei*. Recently, we obtained five monoclonal antibodies (mAbs) directed to

the flagella of *C. chauvoei* (Tanaka *et al.*, 1987). Among them, three had a protective effect on mice, but the remaining mAbs did not, even though all reacted with the 56 kDa protein, which corresponds to flagellin.

In the present study, we have analysed the structural and antigenic characteristics of the protective and non-protective antigen on the flagella of *C. chauvoei* using these mAbs.

Methods

mAbs. Hybridomas producing mAbs against the flagellin of *C. chauvoei* were prepared from splenocytes of BALB/c mice immunized with purified flagella of *C. chauvoei* strain Okinawa and murine myeloma cells (P3-X63-Ag8-U1) as described previously (Tanaka *et al.*, 1987). Hybridoma cells were injected into the peritoneal cavity of pristane-treated BALB/c mice; ascites were used as mAbs. The mAbs used in this study are listed in Table 1. Three mAbs, Mo-41, Mo-62 and Mo-90, showed passive protective effects (90, 50 and 50%, respectively), but the other two, Mo-114 and Mo-2-60, did not (Tanaka *et al.*, 1987).

For the absorption test, mAbs were absorbed with formalin-treated whole cells of *C. chauvoei* strain Okinawa. Wet cells in the late exponential growth phase were added to the mAb solutions at a concentration of 100 mg ml⁻¹, and the suspension was incubated at 37 °C for 2 h and then at 4 °C for another 18 h. The cells were removed by centrifugation at 6500 g for 20 min. This procedure was repeated eight times. The resulting mAbs were used as absorbed mAbs. Mouse ascite induced by myeloma cells was used as a control and designated as P3U1.

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Abbreviations: HRPO, horseradish peroxidase; mAb, monoclonal antibody.

Table 1. Characteristics of mAbs directed to the flagellin of *C. chauvoei*

mAb	Passive protection test*	H-agglutination titre	Immobilization titre
Mo-41	Protective	25600	2560
Mo-62	Protective	25600	2560
Mo-90	Protective	25600	2560
Mo-114	Non-protective	< 100	< 20
Mo-2-60	Non-protective	< 100	< 20

* Mice were injected intraperitoneally with mAbs and challenged intramuscularly with spores of *C. chauvoei* strain Okinawa (100 m.l.d.) (Tanaka *et al.*, 1987).

H-agglutination test and immobilization test. Details of the H-agglutination test and immobilization test were described previously (Tamura & Tanaka, 1984). Formalin-treated whole cells of *C. chauvoei* strain Okinawa were used as the antigen for the H-agglutination test. The immobilization test was performed as follows. Sterilized tubes containing molten CLB broth (Azuchi *et al.*, 1962) with 0.15% agar were placed in a 50 °C water bath, and twofold-diluted ascites were added. The cells were grown anaerobically at 37 °C for 24 h in heart infusion agar (Difco) supplemented with 5% (v/v) defibrinated sheep blood. Single colonies were stab-inoculated into each tube after the soft agar had cooled. The highest dilution of ascites that inhibited the migration of the organism was termed as the immobilization titre of the mAbs.

Immune electron microscopy. *C. chauvoei* strain Okinawa was cultured anaerobically at 37 °C and growth was terminated after 16 h with 0.5% (v/v) formalin during the late exponential growth phase. Freshly prepared formalin-treated cells were washed gently three times with PBS (g l⁻¹: NaCl, 8.0; KCl, 0.2; Na₂HPO₄, 1.15; KH₂PO₄, 0.2; pH 7.2). After centrifugation, the cell pellets were sensitized with 0.2 ml diluted mAbs (1:10 dilution in PBS containing 1%, w/v, BSA) for 30 min at 37 °C. The mixture was washed three times with PBS, resuspended in 0.05 ml 1% (v/v) BSA-PBS, and adsorbed onto carbon-coated grids for 10 min at room temperature. Excess fluid was removed with a filter paper. The grid was then incubated with anti-mouse IgG and IgM goat serum coupled to 5 nm gold colloidal particles (1:10 dilution in 1% BSA-PBS) (E-Y Laboratories) for 30 min at room temperature. The labelled specimens were negatively stained with 1% phosphotungstic acid (TAAB Laboratories Equipment) and observed with a JEOL S-100 transmission electron microscope at 80 kV.

Purified flagella. Purified flagella were prepared from *C. chauvoei* strain Okinawa as described previously (Tamura *et al.*, 1984). Briefly, the formalin-treated whole cells were suspended in 0.1 M-Tris/HCl buffer (pH 8.0) and the cell suspension was homogenized to shear the flagella with a high-speed homogenizer. Deflagellated cells were removed by centrifugation at 6500 g for 20 min and the resulting supernatant was further centrifuged at 77000 g for 90 min. The pellet was suspended in approximately 20 ml 1.0 M-Tris/HCl buffer and mixed with caesium chloride to a final density of 1.35 g cm⁻³. After centrifugation at 73000 g for 24 h, a band appearing at 1.33 g cm⁻³ was harvested and dialysed against 0.05 M-Tris/HCl buffer (pH 7.2).

ELISA with absorbed and unabsorbed mAbs. ELISA was performed as described previously (Tanaka *et al.*, 1987). Briefly, purified flagella were used as the antigen with a protein content of 0.313 µg per well. The wells of micro-ELISA plates were incubated with 100 µl mAbs, or

absorbed mAbs, for 30 min at room temperature. After washing, the wells were incubated with 100 µl horseradish peroxidase (HRPO)-conjugated anti-mouse IgG (heavy plus light chains) goat serum (1:3000 dilution in PBS containing 5% foetal calf serum and 0.05% Tween 20) (Bio-Rad) for 30 min at room temperature. *O*-Phenylene-diamine was used as the substrate, and colour development was terminated with 2.0 M-H₂SO₄. The A₄₉₀ of each well was observed with a SJeia Auto Reader (Sanko Junyaku).

Competitive binding assay. The competitive binding assay was performed as described previously (Tanaka *et al.*, 1987). Briefly, the mAbs were labelled with HRPO by the method of Nakane & Kawaoi (1974). The competing antibody was incubated with the antigen for ELISA, as described above, for 4 h at room temperature, washed six times, and HRPO-labelled mAbs were then added. The percentage competition was determined by the formula $100(A - n)/(A - B)$, where *A* is the A₄₉₀ in the absence of competing antibody, *B* is the A₄₉₀ in the presence of homologous antibody and *n* is the A₄₉₀ in the presence of competitor.

Cyanogen bromide (CNBr) cleavage. CNBr cleavage of flagellin was performed in 70% (v/v) formic acid with an equal amount of CNBr to purified flagella according to the method of Parish & Ada (1969). The cleavage mixture was then diluted with distilled water and lyophilized.

Electrophoresis. SDS-PAGE was performed with a Mini-slab gel apparatus (Bio-Rad) by the method of Laemmli (1970). Purified flagella in sample buffer were stacked in polyacrylamide gels and separated in 12% polyacrylamide at 200 V for 45 min. A low-molecular-mass calibration kit (Pharmacia) was used as the molecular mass standard. Gels were stained with Coomassie brilliant blue R-250.

For CNBr-cleaved flagellin, urea-SDS-PAGE was carried out according to the method of Swank & Munkres (1971). Peptides produced by CNBr cleavage were separated in 12.5% polyacrylamide gels containing 8 M-urea. Electrophoresis was carried out at 100 V for 20 h. Fragments of highly purified myoglobin prepared by cleavage with CNBr were used as molecular mass standards, and peptides were stained with Coomassie brilliant blue R-250.

Immunoblotting assay. Proteins or peptides were electrophoretically transferred to nitrocellulose membranes at 40 V for 2 h with a Trans-blot apparatus (Bio-Rad) by the methanol-Tris/glycine system of Towbin *et al.* (1979). After being probed with mAbs or anti-Okinawa strain mouse serum, the strips were washed, incubated for 1 h with HRPO-conjugated anti-mouse IgG goat serum, and washed again. The strips were developed by using HRP colour development reagent (containing 4-chloro-1-naphthol) (Bio-Rad).

Results

Characteristics of mAbs

Table 1 summarizes the characteristics of the mAbs used. Investigations on the serological reactivity of anti-flagella antibodies were carried out with five mAbs reacting with epitopes on flagella of *C. chauvoei*. In H-agglutination and immobilization tests, the mAbs could be divided into two groups. High titre was observed only with the three protective mAbs, each having a similar titre. In contrast, serological reactivity was absent in the two non-protective mAbs. The immobilization titres were approximately 10-fold lower than the corresponding titres of H-agglutination.

Fig. 1. Immune electron microscopy of *C. chauvoei* strain Okinawa. Whole cells were incubated with mAbs Mo-41 (a) and Mo-114 (b), and processed for immune electron microscopy as described in Methods. Bars, 1 μ m.

Immune electron microscopy

The location of the epitopes on flagella of formalin-treated whole cells was investigated by immune electron microscopy. When mAb Mo-41, which showed a protective effect, was used as the primary antibody, gold particles were observed specifically on the surface of flagellar filaments (Fig. 1a). mAbs Mo-62 and Mo-90, which also had a protective effect, showed the same behaviour as Mo-41. On the contrary, no noticeable association of gold particles with the flagella was observed in the preparations of Mo-114 (Fig. 1b) and Mo-2-60, which did not show a protective effect, or with P3U1 (control). There were no mAbs that specifically labelled the tips or broken pieces of the flagella.

Fig. 2. ELISA reactivity of mAbs directed to the flagellin of *C. chauvoei*. ●, Unabsorbed mAbs; ○, mAbs absorbed with formalin-treated whole cells of strain Okinawa. The numbers within the boxes indicate mAbs.

ELISA reactivity of mAbs

ELISA was used to confirm the location of epitopes recognized by protective mAbs on the surface of flagellar filaments. The protective mAbs, Mo-41, Mo-62 and Mo-90, reacted with surface-exposed epitopes on *C. chauvoei* flagella, as shown by absorption with formalin-treated whole cells of *C. chauvoei* possessing flagella. This resulted in a substantial reduction of >0.5 in maximum A_{490} in ELISA titre (Fig. 2). When mAb Mo-62 was absorbed with whole cells, a much larger reduction in ELISA titre was obtained. In contrast, when the non-protective mAbs, Mo-114 and Mo-2-60, and P3U1 (control) were absorbed with whole cells, the ELISA titres obtained were similar to unabsorbed mAbs.

Competitive binding assay

Four mAbs, Mo-41, Mo-62, Mo-90 and Mo-114, were labelled with HRPO and used for a competitive binding assay. Percentage inhibition curves of diluted competitor are shown in Fig. 3. Reciprocal competitions were observed among Mo-41, Mo-62 and Mo-90. These protective mAbs were, however, not blocked with the other two non-protective mAbs. Moreover, non-protective mAb Mo-114 was blocked only by homologous antibody.

Fig. 3. Competitive inhibition of binding of HRPO-labelled mAbs to flagellin of *C. chauvoei* in the presence of excess concentrations of unlabelled mAbs. ●, Mo-41; ■, Mo-62; ▲, Mo-90; ○, Mo-114; □, Mo-2-60. The numbers within the boxes indicated HRPO-labelled mAbs.

Electrophoretic analysis of purified flagella

The protein profiles of purified flagella were obtained by SDS-PAGE (Fig. 4a). A major protein band with a molecular mass of 56 kDa, corresponding to flagellin, and at least two minor protein bands with molecular masses of approximately 200 kDa were found in purified flagella. The location of flagellin epitopes was investigated by CNBr cleavage of purified flagella. Urea-SDS-PAGE of purified flagella cleaved by CNBr showed major peptides with apparent molecular masses of 33, 20 and 6 kDa (Fig. 4b). There was no cleavage of purified flagella upon incubation in formic acid alone (data not shown).

Immunoblotting assay

The location of the epitopes recognized by mAbs was investigated by an immunoblotting assay. Fig. 5 shows the typical reaction of mAbs; serum from mice immunized with whole cells of *C. chauvoei* strain Okinawa was used as a control. Protective and non-protective mAbs, Mo-41, Mo-62 and Mo-114, reacted with the 56 kDa protein band, and weakly with a 120 kDa band and two

Fig. 4. Electrophoretic analysis of purified flagella with SDS-PAGE (a) and CNBr-cleaved flagellin with urea-SDS-PAGE (b) stained with Coomassie brilliant blue R-250.

Fig. 5. Immunoblotting analysis of mAbs directed to the flagellin of *C. chauvoei* in SDS-PAGE (a-d) and CNBr-cleaved flagellin in urea-SDS-PAGE (e and f). (a) Mo-41; (b) Mo-62; (c and e) Mo-114; (d and f) mouse anti-strain Okinawa serum.

or more bands of approximately 200 kDa with purified flagella in SDS-PAGE (Fig. 5a, b, c). mAbs Mo-90 and Mo-2-60 showed a similar reaction pattern although the reactions were weak (data not shown). Anti-Okinawa strain serum, in comparison with the mAbs, also showed the same reaction pattern. The reaction of mAbs with CNBr-cleaved flagellin was also examined. Only Mo-114 reacted with three peptides of CNBr-cleaved flagellin

(Fig. 5e). In contrast, the other mAbs result in a loss of the immunoblotting reaction (data not shown). With CNBr-cleaved flagellin in urea-SDS-PAGE, however, anti-Okinawa strain serum reacted solely with a fragment of 33 kDa.

Discussion

Flagella of *C. chauvoei* are organelles which help to induce immune resistance mechanisms, as demonstrated by the mouse protection test (Tamura *et al.*, 1984). Flagellar antibodies appear to exert their protective effect by opsonic activity such that opsonized *C. chauvoei* are eliminated by polymorphonuclear leucocytes (Tamura & Tanaka, 1984, 1987). Flagella have, therefore, been considered as one of the candidates for vaccines against blackleg in cattle and sheep. Our previous study showed that three mAbs directed to the flagellin of *C. chauvoei*, Mo-41, Mo-62 and Mo-90, had protective effects in mice (Tanaka *et al.*, 1987). The present study was aimed at elucidation of the protective antigen on the flagella of *C. chauvoei* using these mAbs.

Immunogold-labelled mAbs have been used to visualize epitopes on the flagella of *C. chauvoei*. Protective mAbs specifically labelled the surface of the flagellar filament, while non-protective mAbs did not. These results indicate that the epitopes recognized by the protective mAbs are surface-exposed on the flagellar filament of *C. chauvoei*, and those of non-protective mAbs are not surface-exposed; this could be explained by an inaccessibility of non-protective mAbs to their epitopes. Inadequate exposure of non-protective epitopes on the surface of the flagellar filament is confirmed by the absence of H-agglutination and immobilization activity with non-protective mAbs. Similar phenomena have been reported in *Campylobacter jejuni* with the mAbs directed to the internal epitopes on flagella (Newell, 1986).

These findings were also confirmed by absorption tests of ELISA reactivity. After absorption with whole cells of *C. chauvoei* strain Okinawa possessing flagella, ELISA reactivities of the protective mAbs were significantly reduced compared with those of the unabsorbed mAbs, indicating that protective epitope was exposed on the surface of the flagellar filament.

To ascertain whether these three protective mAbs reacted with the same epitope, a competitive binding assay was performed. When protective mAbs were conjugated to HRPO, competition was observed only among protective mAbs, indicating that their epitopes are identical or topographically overlapping. In contrast, non-protective epitopes are obviously in a position separate from the protective epitope, and each epitope of the non-protective mAbs is topographically separated

from remaining epitopes. On the basis of these results, we have defined three distinguishable epitopes on the flagellin of *C. chauvoei*.

The epitopes participating in protection, when coupled with our previous observations (Tanaka *et al.* 1987), were species-specific and surface-exposed on the flagellar filament. Interestingly, however, the epitopes of the non-protective mAbs were cross-reactive and not surface-exposed implying that, in this case, the non-protective epitopes may carry conserved primary sequences among various clostridia. ELISA reactivity with non-protective mAbs also suggested that the conserved sequence was internally located in the flagellar filament. Logan & Trust (1986) reported that the flagella of *C. jejuni* have both internal and surface-exposed epitopes, and suggest that the immunodominant surface-exposed epitopes are serospecific, and much of the conserved sequence is internally located in the native flagellar filament. Epitopes in polymeric flagellin also play a major role in the serospecificity of *Salmonella* flagella (Ibrahim *et al.*, 1985).

In immunoblotting analysis of purified flagella, all five mAbs reacted with several proteins, showing one clear band of 56 kDa, which corresponded to the flagellin monomer, and at least three weak bands ranging from 120–200 kDa, which are polymeric forms of the flagellin monomer, as described previously (Tanaka *et al.*, 1987). Subsequently, the location of epitopes recognized by the mAbs was investigated using CNBr-cleaved flagellin. Coomassie brilliant blue-stained urea-SDS-PAGE gels of *C. chauvoei* flagellin cleaved by CNBr displayed major peptides with apparent molecular masses of 33, 20 and 6 kDa. The most important result in this study was the observation that the protective mAbs did not react with CNBr-cleaved peptides of flagellin in the immunoblotting analysis, while the non-protective mAb, Mo-114, did. These results suggest that protective mAbs may recognize conformational epitopes derived from the secondary structure of flagellin. On the other hand, the non-protective Mo-114 reacted with three peptides of flagellin cleaved by CNBr, indicating that at least one non-protective mAb may recognize a linear, and conformation-independent epitope in the flagellin. As yet we are unable to define the protective or non-protective epitopes on the flagellin molecule. The identification of protective conformational epitopes in the flagellin of *C. chauvoei* has prompted us to consider the importance of such a determinant as a component in the development of a vaccine that would be effective against blackleg disease. Further studies on the location of protective epitopes that involve the use of these anti-flagellar mAbs are necessary in order to elucidate the native form of the protective antigen on the flagella of *C. chauvoei*.

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