Peptides ¹⁴VIDLL¹⁸ and ⁹⁶FEAAAL¹⁰¹ defined as epitopes of antibodies raised against amino acid sequences of enterotoxigenic *Escherichia coli* colonization factor antigen I fused to *Salmonella* flagellin

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Antibodies raised against four hybrid *Salmonella* flagellins carrying amino acid sequences derived from the fimbrial subunit of the colonization factor I antigen (CFA/I) of enterotoxigenic *Escherichia coli* (ETEC), i.e. hybrid flagellins Fla I (aa 1–15), Fla II (aa 11–25), Fla III (aa 32–45) and Fla IV (aa 88–102), were not able to inhibit the *in vitro* binding of CFA/I-expressing ETEC bacteria to enterocyte-like Caco-2 cells. However, one of the hybrid flagellins (Fla II) was recognized by a previously described anti-CFA/I subunit mAb (S-CFA/I 17:8) which was able to block adhesion of CFA/I-expressing bacteria to Caco-2 cells and to bind to the amino acid sequence ¹⁵IDLLQ¹⁹ of the CFA/I fimbrial subunit. Pepscan analysis of antibodies raised against the hybrid flagellins Fla II and Fla IV showed that they were specific for the sequences ¹⁴VIDLL¹⁸ and ⁹⁶FEAAAL¹⁰¹, respectively, of the CFA/I fimbrial subunit. Thus, the discrepancy in the abilities of the anti-Fla II serum and the mAb S-CFA/I 17:8 to block binding might be ascribed to their slightly different fine specificity for epitopes.

Keywords: enterotoxigenic *Escherichia coli*, CFA/I fimbriae, flagellin, *Salmonella*, monoclonal antibodies

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common causes of acute diarrhoea in children in developing countries and in travellers who visit ETEC-endemic areas (Black, 1990, 1993). Adherence of bacteria to the epithelial surface of the small intestine and delivery of toxins are the two most important events in ETEC pathogenesis. Colonization of the human small intestine by ETEC involves several antigenically different fimbrial or non-fimbrial adhesins collectively termed colonization factors (CFs), but individually called either colonization factor antigens (CFAs), coli surface (CS) antigens or putative colonization factors (PCFs) (Gaastra & Svennerholm, 1996). Since mucosal antibodies to CFs are protective against ETEC disease, these antigens have been included in vaccines (Holmgren &

Svennerholm, 1996). However, protection conferred by immunization with CFs is restricted to those strains expressing homologous adhesins (Svennerholm *et al.*, 1989).

The CFA/I fimbria is one of the most frequently found CFs in epidemiological studies and also one of the best characterized at the molecular level. It has a rigid structure with a diameter of 7 nm and is composed of many copies of a single 15 kDa subunit which shares extensive amino acid sequence similarity with the subunits of other CFs, for example CS1, CS2, CS4, CS17 and PCFO166 (McConnell *et al.*, 1989; Gaastra & Svennerholm, 1996). The CFA/I fimbria is polar with a single receptor-binding domain located at the tip of the fimbrial rod (Bühler *et al.*, 1991; Marron & Smyth, 1995).

In contrast to mAbs raised against intact fimbriae, mAbs against dissociated CFA/I subunits were shown to cross-react with several CFs and could inhibit the adhesion of ETEC expressing homologous and heterologous CFs to enterocyte-like Caco-2 cells and isolated human jejunal enterocytes (Rudin *et al.*, 1994, 1996). One of these

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Abbreviations: CFA, colonization factor antigen; ETEC, enterotoxigenic *Escherichia coli*.

mAbs, S-CFA/I 17:8, bound to a continuous epitope of the CFA/I subunit composed of the amino acid sequence ¹⁵IDLLQ¹⁹ (Rudin & Svennerholm, 1996). However, immunization with a synthetic peptide corresponding to the first 25 N-terminal amino acids of the CFA/I subunit did not generate antibodies with the required specificity to block the adhesion of CFA/I-expressing bacteria to Caco-2 cells (Rudin & Svennerholm, 1996).

Attenuated *Salmonella* strains can induce a broad immune response after oral administration and represent a convenient and low-cost alternative means of presenting heterologous antigens (Hoiseth & Stocker, 1981). Moreover, the insertion of heterologous linear epitopes in *Salmonella* flagellin has been shown to induce humoral immune responses against several antigens, such as cholera toxin (Newton *et al.*, 1989), hepatitis B virus surface antigen (Wu *et al.*, 1989), influenza virus haemagglutinin (McEwen *et al.*, 1992) and streptococcal M protein (Newton *et al.*, 1991). Purified hybrid *Salmonella* flagellins carrying linear epitopes of the CFA/I fimbria have also been shown to induce antibodies against the CFA/I fimbrial subunit after parenteral immunization of mice (Luna *et al.*, 1997).

In this study we analysed mouse antisera against hybrid flagellins with CFA/I-derived amino acid inserts for inhibition of binding to Caco-2 cells. In addition, the epitope specificity of the CFA/I-specific antibodies raised against the hybrid flagellins was evaluated by the Pepscan technique.

METHODS

Bacterial strains and plasmid. The flagellin-negative strain *Salmonella dublin aroA* SL5928 was used as a carrier of plasmids encoding the hybrid flagellins (Newton *et al.*, 1989). The plasmid pLS408 has a deletion of a 48 bp *EcoRV* fragment in the central hypervariable region of the *fliC* (H1-d) flagellin-encoding gene. Therefore, this plasmid contains a unique *EcoRV* restriction site between two codons of the flagellin gene, into which in-frame insertions of oligonucleotides encoding CFA/I sequences could be introduced. Construction and characterization of the *Salmonella* strains harbouring the recombinant pLS408-derived plasmids were carried out as previously described (Luna *et al.*, 1997). The flagellin encoded by plasmid pLS408 was referred to as Fla 30. The flagellins containing CFA/I-derived sequences were named Fla I (aa 1–15), Fla II (aa 11–25), Fla III (aa 32–45) and Fla IV (aa 88–102). For flagellin purification, the *Salmonella* strains were grown at 37 °C in Luria-Bertani broth (Difco) supplemented with 25 µg ampicillin ml⁻¹.

The ETEC strains 258909-3 (CFA/I, O128:H?, ST+/LT+) (Gothefors *et al.*, 1985) and the corresponding CFA/I-negative mutant 258909-3M (Lopez-Vidal *et al.*, 1988) were used in assays of inhibition of binding to Caco-2 cells and immunoelectron microscopy. The ETEC strains were grown on Casamino acids/yeast extract agar plates (CFA agar) at 37 °C overnight (Evans *et al.*, 1979).

Flagellin and CFA/I fimbriae preparations. The different flagellins were purified by acid cleavage, as described by Ibrahim *et al.* (1985). The hybrid flagellin preparations were analysed by SDS-PAGE before immunization to control for

any possibility of degradation of the protein. Protein concentrations were determined by the Lowry method. The CFA/I fimbriae were purified essentially as described by Evans *et al.* (1979). Briefly, bacteria grown on CFA agar were homogenized with a blender and centrifuged at 12000 g for 20 min. The cell-free supernatant fraction was ammonium-sulphate-precipitated (at 20 and 40% saturation), and after centrifugation at 12000 g and dialysis, the pellet was further purified by gel filtration in a DEAE-Sephadex column or by ultracentrifugation in a CsCl gradient (density 1.3 g cm⁻³). The concentration of the fimbriae was determined by an inhibition ELISA using a highly purified lyophilized fimbrial preparation as a reference (Lopez-Vidal *et al.*, 1988).

Antibody preparations. mAbs against CFA/I subunits, i.e. mAbs S-CFA/I 17:8 (144 µg IgG1 ml⁻¹) and S-CFA/I 5:6 (150 µg IgG1 ml⁻¹) had previously been produced against guanidine-hydrochloride-dissociated subunits of CFA/I fimbriae (Rudin *et al.*, 1994). The anti-peptide antiserum was previously raised in rabbits against a synthetic CFA/I peptide consisting of the first 25 N-terminal amino acids of the subunit (Rudin & Svennerholm, 1996). Antisera against each of the modified flagellins were raised in female BALB/c mice after four intraperitoneal immunizations with 10 µg purified flagellin (Luna *et al.*, 1997). The first dose was given in complete Freund's adjuvant, and 3 weeks later a second dose was administered in incomplete Freund's adjuvant. Ten days later two additional doses without adjuvant were given with a 4 d interval. The animals were sacrificed 1 week after the last immunization and blood was drawn by heart puncture. All sera were incubated with a CFA/I-negative strain (258909-3M) and heat-inactivated before use, as previously described (Rudin & Svennerholm, 1996). Immunization of BALB/c mice with isolated hybrid flagellins resulted in the production of specific IgG responses against CFA/I fimbrial subunits (Luna *et al.*, 1997). The CFA/I-subunit-specific IgG titres generated with hybrid flagellins Fla II and Fla IV were higher than those generated with hybrid flagellins Fla I and Fla III (1/6400 and 1/1600 versus 1/100 and 1/800, respectively). No CFA/I-specific antibody titre was detected in antiserum raised against *Salmonella* flagellin without an insert (Fla 30) (Luna *et al.*, 1997).

Agglutination tests. A 10 µl sample of a bacterial suspension (10¹⁰ c.f.u. ml⁻¹) of each recombinant *S. dublin* strain expressing flagellin with or without an insert and of the flagellin-negative strain was mixed with the same volume of undiluted mAbs or antiserum on a glass slide at room temperature. The reaction was considered positive when agglutination was macroscopically visible within 2 min. The bacterial strains mixed with saline served as parallel negative controls.

Inhibition of adhesion to Caco-2 cells. Caco-2 cells were grown for 14–16 d in Dulbecco's modified Eagle's medium containing foetal calf serum (10%, v/v) and glutamine (1%, w/v) in eight-well chamber slides (Nunc) in 7% CO₂ at 37 °C. A suspension of 10⁷ bacteria ml⁻¹ in culture medium containing 0.5% D-mannose was mixed with an equal amount of diluted antiserum and incubated at room temperature for 20 min. The mixture was then added to the Caco-2 cells, washed with Eagle's medium and incubated at 37 °C for 3 h. After five washes, cells were fixed in methanol, stained with 10% (v/v) Giemsa and examined by oil-immersion light microscopy to determine bacterial adherence. The percentage of epithelial cells with at least one adhering bacterium was determined by counting 10 randomly chosen microscopical fields with approximately 100 cells per field. Each antiserum was tested at least in duplicate and all assays were performed in a blind manner.

Table 1. Reactivity in ELISA of mAbs and sera against CFA/I fimbrial subunits and a synthetic N-terminal CFA/I peptide with hybrid flagella bearing CFA/I amino acid sequences, and their capacity to agglutinate *S. dublin* strains expressing hybrid flagellins

Flagellins bearing CFA/I-derived sequences are Fla I (aa 1–15), Fla II (aa 11–25), Fla III (aa 32–45) and Fla IV (aa 88–102). Fla 30 is flagellin without an insert. ELISA titres are means of duplicate determinations. Only positive agglutination results are indicated, and are graded as weak (+) or strong (++) reactions.

Antibody	ELISA titre (agglutination)				
	Fla 30	Fla I	Fla II	Fla III	Fla IV
mAbs					
mAb S-CFA/I 17:8	<20	<20	2700 (++)	<20	<20
mAb S-CFA/I 5:6	<20	<20	<20	<20	<20
Antisera					
Anti-peptide*	<20	100 (+)	670 (++)	<20	<20
Anti-CFA/I subunit†	<20	60	730 (++)	490 (++)	760 (++)

* Rabbit antiserum against peptide aa 1–25 of the protein sequence of the CFA/I fimbrial subunit.

† Mouse antiserum against CFA/I fimbrial subunits.

ELISA. When antisera against the synthetic CFA/I subunit peptide and CFA/I fimbrial subunits as well as the anti-CFA/I mAbs were tested for reactivity with purified hybrid flagellins, polystyrene microtitre plates (Dynatech) were coated with flagellin preparations in PBS (0.145 M sodium chloride, 0.15 M sodium phosphate) ($1 \mu\text{g ml}^{-1}$) and incubated at 37 °C overnight. Conversely, when determining the reactivity of antisera against hybrid flagellins to CFA/I, plates were coated with CFA/I subunits ($0.5 \mu\text{g ml}^{-1}$). After blocking with 0.1 % BSA in PBS at 37 °C for 30 min, antisera were serially diluted in PBS containing 0.1 % BSA and 0.05 % Tween, added to the wells and incubated at room temperature for 60 min. The presence of bound antibodies was then demonstrated by incubating the plates with horseradish-peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and o-phenylenediamine/ H_2O_2 . Titres were determined as the reciprocals of dilutions giving an A_{450} of 0.4 above background (Labsystems Multiscan PLUS) after 10 min. All titrations were performed in duplicate.

Immunoelectron microscopy. Formvar-coated grids (400 mesh) were coated with suspensions of *S. dublin* expressing hybrid flagella for 2 min. The coated grids were placed on a drop with mAb diluted in 1 % BSA/0.05 % Tween/PBS for 15 min and subsequently on drops with gold (10 nm)-labelled goat anti-mouse IgG (BioCell Research Laboratories) diluted 1:30 in 1 % BSA/0.05 % Tween/PBS. After several washings with 1 % BSA in PBS and a final wash with distilled water, the samples were negatively stained with 1 % (w/v) ammonium molybdate (Sigma) and the grids were examined in an electron microscope (JEOL) at 80 kV.

Peptide synthesis. The synthesis of the peptide corresponding to the first 25 N-terminal amino acids of the CFA/I subunit (VEKNITVTASVDPVIDLLQADGNAL) was performed with a Model 430 A Peptide Synthesizer (Applied Biosystems) as previously described (Rudin & Svennerholm, 1996). After synthesis, the peptide was cleaved from the resin and the protecting groups were removed from the amino acids by acidic hydrolysis using anisole and ethanedithiol as scavengers. The peptide composition was confirmed by amino acid analysis, and purity was assessed by reverse-phase high-

pressure liquid chromatography (Rudin & Svennerholm, 1996).

Epitope analysis by Pepscan. A Multipin non-cleavable peptide kit and FMOC (9-fluorenylmethoxycarbonyl)-protected amino acids (Chiron Mimotopes) were used to synthesize sets of linear overlapping hexamer peptides corresponding to the N-terminal (aa 1–52), central (aa 85–114) and C-terminal (aa 121–147) parts of the CFA/I subunit protein by the Geysen pin method (Geysen *et al.*, 1984). The peptides were synthesized on the tips of derivatized polyethylene pins in the configuration of 96-well microtitre plates and then activated with diisopropylcarbodiimide (Merck) and 1-hydroxybenzotriazole (Chiron Mimotopes), as described previously (Rudin & Svennerholm, 1996). Geysen's capture ELISA (Geysen *et al.*, 1984) was utilized with minor modifications. After an initial blocking step, the peptides on the pins were incubated with antisera against hybrid flagellins (1/1000 dilution) at room temperature for 2 h. The pins were then washed and incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG (1/2500 dilution) (Jackson ImmunoResearch) diluted in PBS/1 % sheep serum/0.1 % Tween 20 for 60 min. Subsequently, the chromogenic substrate solution H_2O_2 /ABTS [diammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate)] was added and the A_{405} measured after 25 min on a Labsystem Multiscan Plus. The pins were reused after effective removal of the antibodies by sonication in disruption buffer (1 % PBS, 1 % SDS and 0.1 % 2-mercaptoethanol) at 65 °C for 10 min and hot methanol treatment.

RESULTS

Anti-CFA/I mAbs, anti-CFA/I peptide antibodies and polyclonal anti-CFA/I antibodies react with hybrid flagellins

The mAbs S-CFA/I 17:8 and S-CFA/I 5:6, both obtained from mice immunized with dissociated CFA/I fimbrial subunits, were reacted with intact hybrid flagella expressed on the surface of *S. dublin* strain

Fig. 1. Electron micrograph of immunogold labelling of Fla II flagellum bearing the CFA/I-subunit-derived amino acid insert (aa 11–25) (a) and Fla 30 flagella without insert (b), expressed by recombinant *S. dublin*. Whole bacterial cells expressing flagella were labelled with mAb S-CFA/I 17:8 at a final concentration of $72 \mu\text{g ml}^{-1}$ and negatively stained. Bars, 200 nm.

Table 2. Inhibition of binding of CFA/I-expressing bacteria to Caco-2 cells using antisera against *S. dublin* hybrid flagellins bearing inserted CFA/I amino acid sequences

mAb S-CFA/I 17:8 was used at a final concentration of $14.4 \mu\text{g ml}^{-1}$; all anti-flagellin sera were used in a final dilution of 1:4. Values are means from two separate experiments.

Antibody	Caco-2 cells with adherent CFA/I ⁺ bacteria (% $\pm$ 1 SD)
None	22.5 ± 2.5
mAb S-CFA/I 17:8	2.7 ± 0.45
Anti-Fla 30	21.1 ± 3.18
Anti-Fla I	20.5 ± 0.50
Anti-Fla II	20.6 ± 3.15
Anti-Fla III	24.0 ± 1.00
Anti-Fla IV	23.5 ± 1.67

SL5928 in agglutination tests or with purified hybrid flagellin in ELISA. Both of these mAbs had previously been shown to inhibit the CFA/I-mediated adhesion of ETEC bacteria to Caco-2 cells (Rudin *et al.*, 1994). mAb

S-CFA/I 17:8 reacted specifically with hybrid Fla II flagella and Fla II flagellin (bearing CFA/I aa sequence 11–25) in agglutination tests and ELISA, respectively, whereas no reaction was detected with mAb S-CFA/I 5:6 (Table 1). Rabbit antibodies raised against a synthetic linear peptide containing the first 25 N-terminal amino acids of the CFA/I fimbrial subunit recognized only hybrid Fla I and Fla II flagella and flagellins in agglutination tests and in ELISA, respectively. Mouse polyclonal antibodies generated against dissociated CFA/I fimbrial subunits reacted with hybrid Fla II, Fla III and Fla IV flagellins and flagella in ELISA and in agglutination tests, respectively. However, these anti-CFA/I subunit antibodies did not react with Fla I flagella in agglutination tests and only weakly with hybrid Fla I flagellin in ELISA. The agglutination tests strongly indicated that the amino acid sequence inserts corresponding to regions of the CFA/I fimbrial subunit were surface-exposed on all recombinant *Salmonella* strains. Further evidence for surface exposure of the inserted CFA/I amino acid sequence in Fla II flagella was obtained from immunogold labelling experiments with mAb S-CFA/I 17:8. Very strong labelling was observed along the flagellar shafts of the *S. dublin* strain expressing hybrid Fla II flagella after reaction with mAb S-CFA/I 17:8, whilst no labelling was detected when the

Fig. 2. Pepscan analysis of epitope reactivity of antisera against hybrid flagellins Fla II and Fla IV bearing CFA/I subunit amino acid sequences. ELISA results (expressed as A_{405} values) from testing anti-Fla II and anti-Fla IV mouse antisera against 94 hexamer peptides corresponding to the N-terminal (aa 1–47), central (aa 85–109) and C-terminal (aa 121–142) parts of the CFA/I fimbrial subunit sequence. The indicated peptide numbers refer to the first amino acid in the sequence of each hexapeptide on each pin. (a) Antiserum against hybrid flagellin Fla II expressing region II (aa 11–25) and (b) antiserum against hybrid flagellin Fla IV expressing region IV (aa 88–102), of the CFA/I fimbrial subunit sequence. Both antisera were diluted 1/1000.

S. dublin strain expressing flagella without insert (Fla 30) was used (Fig. 1).

Anti-hybrid flagellin antibodies do not inhibit binding of CFA/I-expressing bacteria to Caco-2 cells

In a previous study we found that none of the anti-hybrid flagellin antisera could inhibit haemagglutination promoted by ETEC expressing CFA/I fimbriae (Luna *et al.*, 1997). As a more physiological alternative to inhibition of haemagglutination we attempted to block the binding of CFA/I-expressing bacteria to Caco-2 enterocyte-like cells. No reduction in the numbers of Caco-2 cells with adhering bacteria was observed in experiments carried out with antisera from mice immunized with the four different hybrid flagellins (Table 2). As a positive control, mAb S-CFA/I 17:8 inhibited the adherence of CFA/I-expressing bacteria to Caco-2 cells by approximately 90%, confirming previously published results (Rudin *et al.*, 1994).

Pepscan analysis defines the CFA/I epitope specificity of antisera raised with hybrid *Salmonella* CFA/I flagellins

To investigate whether the failure of the anti-Fla II antiserum to inhibit adhesion of CFA/I-expressing bacteria (Table 2) might be due to a different epitope specificity from mAb S-CFA/I 17:8, Pepscan analyses were performed to identify linear CFA/I epitopes

recognized by the antibodies raised against the hybrid flagellins. In contrast to mAb S-CFA/I 17:8, which reacted specifically with the peptide sequence $^{15}\text{IDLLQ}^{19}$ (Rudin & Svennerholm, 1996), the antibodies raised against purified hybrid flagellin Fla II bound strongly only to peptides 13 and 14 having the amino acid sequence $^{14}\text{VIDLL}^{18}$ in common (Fig. 2). Anti-Fla IV antiserum, which also possessed high antibody titres against CFA/I fimbrial subunits, was similarly submitted to Pepscan analysis. It reacted only with a short peptide sequence constituted by the hexapeptide $^{96}\text{FEAAAL}^{101}$. Antisera against the other hybrid flagellin constructs, Fla I and Fla III, failed to react with specific linear peptides. Higher concentrations of anti-Fla I and anti-Fla III antisera were subsequently used, but the background increased dramatically and no specific reaction could be observed. Control non-immune serum showed a low background with no pre-existing specific antibodies to the peptides (data not shown).

DISCUSSION

Long-term protection against ETEC disease requires the induction of intestinal IgA against the various ETEC fimbriae to prevent colonization, which is the first event in ETEC pathogenesis (Holmgren & Svennerholm, 1996). Although the majority of the CFA fimbrial epitopes are conformational, a few linear epitopes have been identified after immunization of monkeys with isolated CFA/I fimbrial subunits (Cassels *et al.*, 1992).

Antibodies against one linear epitope located in the N-terminal part of the CFA/I subunit have been shown to interfere with the adherence of fimbriated bacteria to Caco-2 cells (Rudin *et al.*, 1994). However, the development of peptide-based vaccines has been hampered by the low immunogenicity of synthetic peptides, particularly if administered on mucosae.

An alternative means of presenting linear epitopes to the mucosal immune system could be to use live recombinant *Salmonella* strains expressing hybrid flagellins (Newton *et al.*, 1989). Such engineered strains have been shown to elicit humoral, mucosal and cellular immune responses after oral or parenteral immunization of animals (McEwen *et al.*, 1992; Newton *et al.*, 1989, 1991; Wu *et al.*, 1989; Verma *et al.*, 1995a, b). The successful application of this epitope delivery system to fimbrial antigens would require the cloning and expression of epitope(s) close to the receptor-binding site. In addition, the induced antibodies would need to display the appropriate specificities to bind and neutralize the fimbrial subunit in its polymeric form.

Previous reports suggested that only the terminal structural subunit of the CFA/I fimbriae exposes a receptor-binding domain (Bühler *et al.*, 1991; Marron & Smyth, 1995). This receptor-binding site probably includes the amino acids ¹⁵IDLLQ¹⁹ since the mAb S-CFA/I 17:8, specific for this epitope, was very efficient in inhibiting adhesion of ETEC bacteria to Caco-2 cells and human enterocytes (Rudin & Svennerholm, 1996; Rudin *et al.*, 1996). Thus, the binding of mAb S-CFA/I 17:8 to hybrid flagellin Fla II, as shown both by agglutination tests and by immunogold labelling, indicated clearly that the expressed epitope had the right conformation for the mAb to bind strongly and was surface-exposed on the recombinant flagella. In addition, antibodies raised against the hybrid flagellin could recognize the CFA/I subunit in ELISA or immunoblots (Luna *et al.*, 1997). Therefore, it was surprising that the anti-Fla II antibodies were not able to block binding of CFA/I-expressing bacteria to the Caco-2 cells. However, this result was in line with previous experiments showing lack of inhibition of haemagglutination using these antisera (Luna *et al.*, 1997).

Our findings using Pepscan analysis suggest that exposure of a sequence on the surface of the *Salmonella* flagellin does not ensure production of antibodies against all possible epitopes of that sequence. Thus, polyclonal antibodies against the Fla II insert (aa 11–25) reacted with only one linear epitope, composed of the sequence ¹⁴VIDLL¹⁸. The same epitope specificity was obtained in rabbits immunized with a synthetic peptide corresponding to the first 25 N-terminal amino acids of the CFA/I fimbrial subunit and, similarly to our results, no significant inhibition of Caco-2 cell binding was found using this anti-peptide antiserum (Rudin & Svennerholm, 1996). Therefore, the different epitope specificity of the anti-Fla II serum compared with that of mAb S-CFA/I 17:8 could be an explanation for the lack of inhibition of binding of CFA/I fimbriated bacteria to

the Caco-2 cells. Interestingly, the results obtained using two different ways (hybrid flagellin and synthetic peptide) of presenting the amino acid sequence in different animal species indicated that the sequence ¹⁴VIDLL¹⁸ is the major linear epitope when the CFA/I N-terminal peptide is presented to the immune system. With both immunization procedures, a minor change of the epitope specificity, in relation to the sequence recognized by the mAb S-CFA/I 17:8, seems to cause a significant alteration in the affinity of the antibodies to the native protein. Such different epitope specificities may also explain a previous report of antibodies raised against an epitope of hen egg-white lysozyme fused to *E. coli* flagellin, which failed to bind the same sequence in the native protein (Kuwajima *et al.*, 1988).

Immunization of mice with the purified hybrid flagellin Fla IV (aa 88–102) also resulted in the generation of antibodies against a single linear epitope, the sequence ⁹⁶FEAAAL¹⁰¹. As far as we know, this is the first report of B-cell epitope specificity analysis of antibodies raised against sequences inserted in *Salmonella* flagellin and, in both of the antisera tested, the same restricted epitope specificity was obtained. We hypothesize that the conformation of the recombinant flagellins might favour the selection and presentation of unique sequences in the insert by antigen-presenting cells. Additional fine-specificity analysis of antisera raised against hybrid flagellins fused to other antigens may demonstrate if such strong preference for single linear epitopes extends to other flagellin constructs.

The analysis of the epitope specificity of serum antibodies of mice immunized with purified hybrid flagellin Fla II suggests that attempts to develop oral live *Salmonella* vaccines would probably not generate a mucosal immune response able to block adhesion of CFA/I-expressing bacteria to enterocytes. In contrast, previous attempts based on the expression of linear epitopes in the *Salmonella* flagellin system have succeeded in generating antibodies able to bind the M protein of *Streptococcus pyogenes* and the haemagglutinin of the influenza virus, leading to partial protection upon challenge (Newton *et al.*, 1991; McEwen *et al.*, 1992). As regards the CFA/I fimbrial subunit, a different conformation of the insert fused to the flagellin might be necessary for the generation of protective antibodies. The incorporation of amino acid residues bordering the heterologous insert in flagellin has been shown to affect the host immune response to the heterologous antigen (Verma *et al.*, 1995a). A similar approach may also be applied to the CFA/I subunit sequence as a way to modify the conformation of the insert and, consequently, the specificity of the antibodies directed against the flagellin insert.

Although most of the previous studies using the *Salmonella* flagellin expression system have demonstrated the generation of serum antibodies against the heterologous insert, the epitope specificities of the antibodies have not been elucidated. Our results suggest that the fine specificity of linear epitopes recognized by anti-

bodies raised against hybrid flagellin constructs may be important in terms of their interaction with the native protein.

ACKNOWLEDGEMENTS

The skilful assistance of Gudrun Wiklund in the purification of fimbriae and of Ella Olofsson in the production of mAbs is gratefully acknowledged, as well as the technical assistance of Marcelo M. Martins, Celso Pereira and Eduardo Camacho. We also thank Ann-Mari Svennerholm for support and valuable discussions, and Anna Helander for expert advice on the electron microscopy. This work was supported by the World Health Organization (WHO), the Swedish Agency for Research Cooperation in Developing Countries (SAREC), the Faculty of Medicine of Göteborg University, and by PADCT and FINEP. M. G. L. received a Fellowship from the Brazilian RHAE programme.

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Received 11 February 1997; revised 10 June 1997; accepted 20 June 1997.