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Linear epitopes of colonization factor antigen I and peptide vaccine approach to enterotoxigenic *Escherichia coli*

FJ Cassels[1], DL Jarboe[2], RH Reid[3], A Lees[4] and CD Deal[5]

[1,2,3]*Department of Gastroenterology;* [5]*Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC 20307;* [4]*Virion Systems Inc, 9610 Medical Center Drive, Rockville, MD 20850, USA*

Enterotoxigenic *Escherichia coli* (ETEC) cause diarrhea in infants and in travelers to developing countries. The bacteria utilize colonization factors (CF) for adherence to intestinal epithelia, then release toxins causing diarrhea. CF are strong immunogens as well as protective antigens. While 20 ETEC CF have been described in the literature, 11 CF are prominent enough to be considered for vaccine targeting. Of this group, six of the members fall into the CFA/I family of CF. Geysen pin (peptide) linear epitope analysis demonstrated that three regions containing linear epitopes exist in CFA/I, and that both B- and T-cell linear epitopes of CFA/I were concentrated at the N-terminus of the protein. We have determined N-terminal sequence of the CFA/I family members not previously sequenced. Comparison of the protein sequence of the six members of the family showed a strong homology up to residue 36. A peptide of 36 amino acids representing a consensus of the six sequences was synthesized and used to immunize animals. The antibody induced to the peptide was reactive to the peptide as well as cross-reactive to each member of the CFA/I family in Western blots. In addition, this antibody agglutinated three of the six members of the CFA/I family when added to whole cells expressing the native CF. We are currently evaluating different carriers and conjugation methods to maximize production of high titer, agglutinating antibody. It is hoped that this and related research will result in an effective and inexpensive cross-reactive and cross-protective ETEC vaccine.

Keywords: linear epitopes; colonization factors; enterotoxigenic *E. coli*; peptide vaccine

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is one of five [6] categories of diarrheagenic *E. coli*. ETEC is a public health problem primarily in developing countries, inducing diarrhea resulting in a high degree of infant mortality and causing diarrhea in travelers to these countries. ETEC utilize colonization factors (CF) to adhere to the small intestinal epithelia where they colonize and express toxins that stimulate fluid secretion. The toxins produced are a heat-labile, multisubunit toxin (LT), and a heat-stable, small peptide toxin (ST); the production of one or both of the toxins defines the strains as ETEC. CF have been identified as distinct antigens by antibody agglutination of whole bacterial cells, immunodiffusion, and by ELISA. Currently 20 distinct colonization factors are recognized [6,14,29].

CF are important vaccine targets due to their role in adherence and because they are highly immunogenic proteins. Current ETEC vaccine efforts concentrate on CF as the principal antigens, whether purified and microencapsulated [22,28], present on killed whole cells [26], expressed on attenuated *Salmonella typhi* [13], or as antigens used to immunize cows, with the bovine antibody then given as a passive vaccine [11,27].

While there are many different distinct ETEC CF described, some CF share properties, leading to groupings into related CF. The relationship may be based on some level of antibody cross-reactivity [20], protein sequence relationship [6], or by common genetic regulation [25]. There currently are two recognized ETEC CF families, the CFA/I family (including CFA/I, CS1, CS2, CS4, CS17, and PCF O166 [6]) and the CS5 family (including CS5 and CS7 [2,8]).

This report examines published and unpublished data on linear B- and T-cell epitopes of CFA/I, the prototypical CFA/I family member, and describes an experimental peptide vaccine approach to ETEC resulting in antibody highly cross-reactive to CF of all the members of the CFA/I family.

Materials and methods

CF purification

CFA/I used in monkey immunizations: Intact CFA/I fimbriae were purified from ETEC strain H10407 (078:H-) as described by Hall *et al* [15] as intact fimbrial fibers. Purified intact CFA/I fibers were dissociated to free subunits in 6 M guanidinium HCl, 0.2 M ammonium bicarbonate, and passed through a 50 000 molecular weight cutoff ultrafiltration membrane (Amicon, Danvers, MA, USA). CF used in Western blot assays: ETEC strains bearing CF from each member of the CFA/I family were harvested, sheared, and fimbriae recovered by ammonium sulfate precipitation of the shearate.

Animal immunization

Three rhesus monkeys were immunized intramuscularly with dissociated CFA/I in complete Freund's adjuvant and

Correspondence: FJ Cassels, Department of Gastroenterology, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA
Current addresses: [2]USAMRMC, MCMR-PLA, Ft Detrick, Frederick, MD 21702; [3]South Mountain Restoration Center, South Mountain, PA 17261; [5]Sexually Transmitted Diseases Branch, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20852, USA
Abbreviations used are as follows: CF, colonization factor; CFA, colonization factor antigen; CS, coli-surface-associated antigen; ETEC, enterotoxigenic *E. coli*; LT, heat-labile toxin; PCF, putative colonization factor; ST, heat-stable toxin.
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subsequently with two injections in incomplete Freund's adjuvant at weekly intervals [4]. Rabbits were immunized subcutaneously with consensus peptide-bovine serum albumin (BSA) using complete (primary immunization) and incomplete (boosts) Freund's adjuvant [5].

Peptide pins for linear epitope analysis

Synthetic peptides covalently linked to polypropylene pins were synthesized according to a variation [4,9] of the method originally described by Geysen [12]. This technique incorporates hardware and software designed at the Walter Reed Army Institute of Research [1]. For B-cell epitope analysis, peptides were synthesized as octamers and remained linked to the resin. For T-cell epitope analysis, the peptides were synthesized as decamers with an additional aspartic acid-to-proline (Asp-Pro) spacer between the pins and the peptides of interest. The Asp-Pro linkage is acid-labile allowing cleavage of the decamers from the pins for T-cell proliferation assays [30]. For a complete description of the B- and T-cell epitope analysis of CFA/I, refer to [4] and [16], respectively.

B-cell epitope peptide pin ELISAs

ELISA assays were performed as previously described [4]. Briefly, to block non-specific binding, the peptide pins were incubated for 1 h at room temperature in a solution of 1% bovine serum albumin in 50 mM sodium phosphate, pH 7.4, 0.1% Tween 20. The monkey sera was diluted in blocking solution and incubated with the pins overnight at 4°C. The pins were washed three times batchwise with 50 mM sodium phosphate, pH 7.4, 0.1% Tween 20 (PBS/Tween), then incubated with alkaline phosphatase-labeled second antibody (Kirkegaard & Perry, Gaithersburg, MD, USA) diluted in blocking solution for 1 h at room temperature. After three more washes with PBS/Tween, pins which had bound antibody were detected by reaction with 0.1% *p*-nitrophenyl-phosphate, in 0.1 M diethanolamine, pH 9.8, 0.02% sodium azide, and 0.01% magnesium chloride for 1 h at room temperature. Development of the ELISA was terminated by removing the pins from the substrate solution, and the absorbance read at 410 nm.

T-cell epitope lymphocyte proliferation

Euthanasia was induced using an overdose of pentothal, then the spleen removed. Single cell suspensions were prepared and washed in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin, streptomycin, L-glutamine, and HEPES buffer (10 mM) (all obtained from Gibco, Gaithersburg, MD, USA), as well as MEM non-essential amino acid solution, MEM amino acids, sodium bicarbonate, and β-mercaptoethanol (Sigma, St Louis, MO, USA). Cell suspensions were adjusted to 10^7 cells per ml in DMEM, and autologous serum was added to yield a concentration of 1.0%. Cells (0.05 ml) were plated in 96-well flat bottom culture plates (Costar, Cambridge, MA, USA) along with 0.05 ml of various dilutions of antigen in DMEM without serum (yielding a 0.5% final concentration of autologous serum) and were incubated at 37°C in 5% CO_2. All cultures were pulsed with 1 μCi [^{3}H]thymidine (25 Ci mmol^{-1}, Amersham, Arlington Heights, IL, USA) on day 4 of culture and were harvested for scintillation counting 6 h later. All lymphocyte proliferation assays were conducted in replicates of four.

Immunoelectron microscopy (IEM)

Suspensions of H10407 (078:H11) were applied to the surface of formvar/carbon-coated grids. Grids were placed on a drop of monkey 2Z2 serum diluted 1/20 in phosphate buffer saline (PBS) with 1% bovine serum albumin (PBS-BSA), incubated for 20 min at room temperture, and washed by placing the grid on successive drops of PBS-BSA. Grids were placed on drops of 10 nm Protein G gold, diluted in PBS-BSA for 20 min. Final washing was carried out on four drops of PBS and two drops of water. Samples were then negatively stained with 2% ammonium molybdate and examined in a Joel Model 100B electron microscope at 80 kV.

Solid phase peptide synthesis

CFA/I family purified CF samples were run on polyacrylamide gels as intact proteins or after formic acid pretreatment. Stained bands from transfers of the gels to PVDF membrane were excised and subjected to automated Edman degradation for N-terminal protein sequence determination [4]. A peptide designed from the consensus of the six members of the CFA/I family was synthesized (sequence VEKNITVTASVDPTIDLLQADGSALPSAVALTYSPA) by solid phase automated FMOC chemistry [5]. Within the N-terminal 36 amino acids of the CF of the six members of the CFA/I family, there is a high degree of identity and homology (83 and 92%, respectively). The consensus of the sequences of the six CF was followed in all positions except position 27, where two serine and two threonine residues resided. Serine was chosen for the consensus peptide due to its presence in CFA/I. An additional amino acid, cysteine, was added to the N-terminus in order to facilitate conjugation. Carrier protein (BSA) was iodoacetylated and the cysteine-peptide immediately added. After conjugation, free peptide was removed by gel filtration [18].

Western blot and bacterial agglutination

Antisera raised to the consensus peptide was assessed for reactivity by immunoblot (Western) analysis of sera tested against fimbriae and consensus peptide-ovalbumin conjugate transferred to nitrocellulose. Intensity of staining was scored visually on a scale of 0 (negative) to 4+ (maximal) value. In addition, sera were tested for the ability of antibody to agglutinate whole cells bearing the native CF by slide agglutination [5].

Results

B-cell epitopes of CFA/I

Sera from rhesus monkeys immunized with CFA/I subunits were tested in the capture ELISA assay. The peptides considered positive were defined as those peaks which clearly stood out above the background and had little or no increase in absorbance compared with the preimmune serum of the same animal [4]. Table 1 summarizes the individual monkey antibody responses to overlapping octapeptides of CFA/I. Eight linear B-cell epitopes were identified by at least a single monkey response, with four regions recog-

Table 1 Linear B-cell epitopes of CFA/I in rhesus monkeys

Sequence position	Number responding	Common region
3–11	1	
11–21	3	DPVIDLLQ
22–29	2	GNALPSAV
32–40	1	
38–45	1	
66–74	2	PQLTDVLN
93–101	2	AKEFEAAA
124–136	2	GPAPT

nized by two monkeys (amino acids 22–29, 66–74, 93–101, and 124–136), and a single region (11–21) recognized by all three monkeys. Five of the eight B-cell epitopes were located in the N-terminus of CFA/I, from residues 1–45. Figure 1 depicts the linear B-cell epitopes with respect to the overall primary sequence of CFA/I.

T-cell epitopes of CFA/I

Spleenic lymphocytes from the three immunized rhesus monkeys were cultured with overlapping decapeptides representing the entire CFA/I sequence in order to determine which segments of the CFA/I protein are able to stimulate proliferation of CFA/I immune primate lymphocytes *in vitro*. All lymphocyte proliferation assays were conducted in replicates of four, and standard deviations of the counts per minute determined. Statistical significance for the proliferative assay was determined using the Student's *t*-test to compare the cpm of quadruplicate wells cultured with the CFA/I peptides to the cpm of wells cultured with a control peptide [16]. Eleven epitopes were recognized by the three monkey responses, with all three monkeys recognizing the peptide at amino acid 133–142, and three other peptides recognized by two different monkeys (Table 2). Responding peptides were located within three distinct regions of the protein (encompassing amino acids 4–35, 72–96, and 126–142, Figure 1).

Immunoelectron microscopy

Serum from each of the three monkeys was tested in IEM on CFA/I bearing H10407. Serum from each of the three monkeys gave a similar result with all showing a labeling of gold particles along the fimbrial shaft. The result of a single individual monkey antibody response (number 2Z2) is depicted in Figure 2.

Table 2 Linear T-cell epitopes of CFA/I in rhesus monkeys

Sequence position	Number responding	Common region
4–13	2	NITVTASVDP
8–17	1	
12–21	2	DPVIDLLQAD
15–24	2	IDLLQADGNA
20–29	1	
26–35	1	
72–81	1	
78–87	1	
87–96	1	
126–135	1	
133–142	3	GNYSGVVSLV

Immunization of rabbits with consensus peptide

Additional protein sequence of CS2, CS4, CS17, and PCF O166 was obtained (Cassels *et al*, manuscript in preparation) and used to derive a consensus sequence. A 36-amino acid peptide based on the consensus, of the sequence VEKNITVTASVDPTIDLLQADGSALPSAVALTYSPA, was synthesized with an N-terminal cysteine, coupled to BSA, and utilized for rabbit immunizations. Immunoblot analysis demonstrated that the peptide conjugate induced a strong antibody response. In addition, the immunoblot analysis demonstrated that antipeptide antibody strongly cross-reacted with each member of the CFA/I family of proteins (Table 3). The four CF most strongly recognized were CS1, CS2, CS4, and PCF O166. In a bacterial slide agglutination assay, the antibody agglutinated ETEC bearing CF of three of the six members (Table 3). The three ETEC strains agglutinated included two of the four CF recognized most strongly by immunoblot analysis, and the strain bearing CFA/I.

Conclusions

Eight B- and eleven T-cell epitopes of CFA/I were recognized by the immune response of three rhesus monkeys immunized with CFA/I subunits. Three combined (at least partially overlapping) B- and T-cell epitope domains are seen in CFA/I: the N-terminal domain (covering amino acids 3–45), the internal domain (66–101), and the C-terminal domain (124–142). All three domains combined account for 65% of the total amino acids of CFA/I. Regions where both B- and T-cell epitopes directly overlap are more limited, with only two regions of more than four amino acids each (amino acids 4–29 and 126–136). Each of these overlapping B- and T-cell epitopes contain at least part of the major immunodominant B-cell epitope (11–21) and the major immunodominant T-cell epitope (133–142). The only section of the CFA/I sequence that contains both immunodominant B- and T-cell epitopes that directly overlap is the portion from amino acids 11–29.

In a precursor to this study, our experience with *E. coli* AF/R1 fimbriae [3] showed that immunization with intact AF/R1 produced little or no response to linear epitopes where sera derived from immunization with AF/R1 dissociated subunits gave a strong response to linear epitopes. Rudin *et al* [23] repeated these findings using CFA/I in mice, and similar results were found with CS31A adhesin [21]. The necessity to denature the intact fimbrial fiber does not appear to be necessary to obtain a response to linear B-cell epitopes for Type 4 fimbriae of *Pseudomonas aeruginosa* [7] and *Neisseria gonorrhea* [10]. While immunization with CFA/I subunits resulted in antibody to linear epitopes, it also yielded antibody binding to intact, native fimbriae as demonstrated by IEM.

This study indicates that an antibody response to a 36-amino acid N-terminal consensus peptide derived from the CFA/I family was immunogenic and stimulated antibodies that are cross-reactive to each member of the family. The consensus peptide differs from the first 36 amino acids of CFA/I

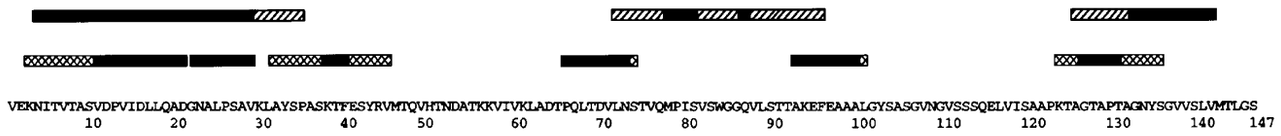

Figure 1 Linear B- and T-cell epitopes of CFA/I as determined by Geysen linear epitope mapping techniques [4,16]. The experimentally determined linear T-cell (uppermost lined bars) and B-cell epitopes (lower cross hatched bars) are depicted above the amino acid sequence of CFA/I. Solid areas within the bars indicate either overlap or two or more monkeys responding to the same amino acids.

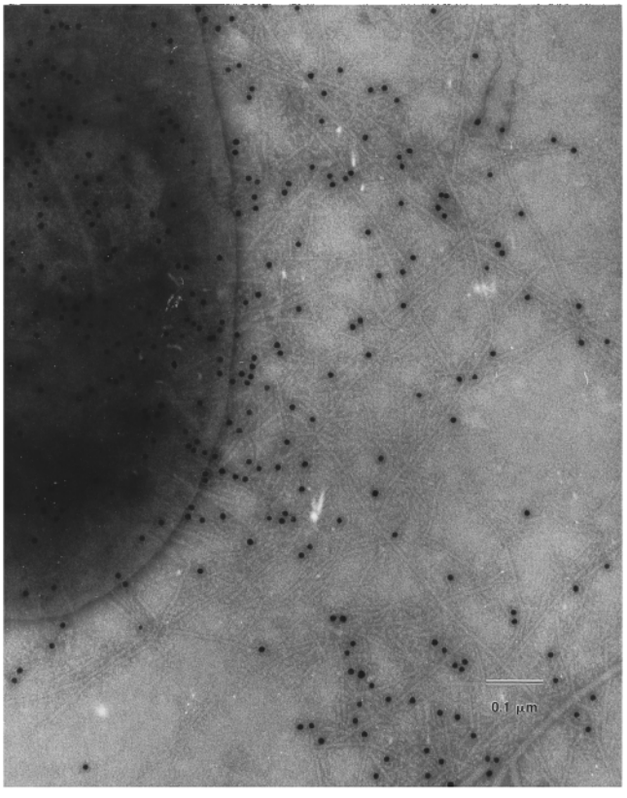

Figure 2 Immunoelectron microscopy of ETEC strain H10407 (O78:H11) labeled with monkey 2Z2 serum raised to CFA/I subunits. See Cassels *et al* [4], for additional micrographs with results of hyperimmune sera of all three monkeys as well as results from preimmune sera.

Table 3 Immunoblot (Western) and slide agglutination results of rabbit antibody to CFA/I family consensus peptide-BSA conjugate tested against purified protein and whole bacterial cells

Antigen[a]	Western blot result[b]	Slide agglutination
CFA/I	++	yes
CS1	++++	yes
CS2	++++	yes
CS4	+++	no
CS17	++	no
PCF O166	+++	no
Peptide-ovalbumin	++++	n.a.[c]

[a]Antigen present on nitrocellulose (Western blot) or on intact bacteria (slide agglutination).
[b]Visual score (0–4+) of Western blot at antibody dilution of 1/5000.
[c]Not applicable.

in four positions (14, 23, 30, and 32). Of these differences only amino acid 32 is a conservative difference (alanine and threonine). Position 14 and 23 are both contained within the combined immunodominant T- and B-cell epitope containing regions. Amino acid 32 is contained in a non-immunodominant combined T- and B-cell epitope containing region, while amino acid 30 is contained within a non-immunodominant T-cell epitope region. It is not known whether the differences between the sequence of CFA/I and the sequence of the consensus peptide would modify the epitopes present to any great extent, but clearly epitopes are present in the consensus peptide. Without further definition of the epitopes contained within the consensus peptide, the contribution of individual amino acids to each epitope and the effect of substitutions on those epitopes is yet to be determined.

Antibodies raised to the consensus peptide are capable of causing the agglutination of whole cells bearing three of the native proteins from which the consensus was derived. It may be possible to obtain agglutination of additional cells bearing CS4, CS17, and PCF O166 by increasing the titer of the antibody (by additional boosting, improved carrier molecule, or by concentration of serum). Also, by selecting for IgM isotype rather than IgG, improved agglutination results may be obtained. Other investigators are studying the linear B-cell epitopes of CFA/I. Rudin *et al* [24] have produced rabbit antibodies to a synthetic peptide representing the N-terminal 25 amino acids of CFA/I. The antibody reacted with CFA/I, CS1, CS2, CS4, and PCF O166 [24]. Synthetic oligonucleotides encoding four regions of the CFA/I molecule were cloned into the *Salmonella* gene coding for flagellin [19]. Three of the regions encoded fell within the N-terminal 45 amino acids of CFA/I. The greatest mouse serum response was to the second region, amino acids 11–25, falling within the section of CFA/I described in this work as containing both immunodominant B- and T-cell epitopes.

Current ETEC vaccines do not attempt to achieve any level of anti-CF cross-reactivity. The B subunit of cholera toxin is included with killed whole ETEC cells for its ability to cross-react with LT [26]. The major emphasis in ETEC vaccines has been on obtaining an immune response to individual CF, not to obtain a cross-reactive, cross-protective response. The CFA/I family discussed herein consists of CFA/I, CS1, CS2, CS4, CS17 and PCF O166. Two recently described colonization factors appear to also fall in the CFA/I family. CS19, identified from a human ETEC strain recovered in India, has a high degree of protein sequence homology to CS17 [14]. An additional colonization factor has been described from ETEC strains isolated from Egyptian children with diarrhea, and identified by antibody cross-reactivity to CS1 and CS17 [17]. If

including the latter newly described CF, 21 ETEC CF have been described to date, with eight of the total being identified as belonging to the CFA/I family [6,14,17,29]. These recent findings illustrate the problem of a large and ever increasing number of ETEC CF, and the necessity for cross-reactive, cross-protective ETEC immunogens.

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