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Genetic fusion of a non-toxic heat-stable enterotoxin-related decapeptide antigen to cholera toxin B-subunit

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A decapeptide highly homologous to the STa *Escherichia coli* heat-stable enterotoxin and to several other heat-stable enterotoxins was fused genetically to the amino-end of the B-subunit of cholera toxin (CTB) and the hybrid protein gene expressed from a *tac*P overexpression system. The STa-related decapeptide used, which was encoded by a synthetic oligodeoxynucleotide, contained a single mutation which substituted a disulfide-linked cysteine by alanine. After its fusion to CTB the decapeptide was able to both react with and to give rise to anti-STa antibodies. Expression of the decapeptide-CTB hybrid by non-toxigenic *Vibrio cholerae* resulted in its full secretion into the extracellular milieu from where it could then be readily purified by single-step affinity chromatography using immobilized GM1 ganglioside. Bacteria producing this non-toxic, immunogenic decapeptide-CTB toxoid might be useful for the development of oral vaccines against diarrhea caused by *E.coli* and other bacteria producing immunologically related heat-stable enterotoxins, and as a source of immunoreagents for methods used to diagnose disease caused by these bacteria.

Hybrid toxoid; Disulfide-dependent enterotoxin epitope; Protein secretion; Enterotoxic diarrhea; Bacterial vaccine

1. INTRODUCTION

Infection by enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrheal disease in infants and children in developing countries and in travellers visiting those places [1]. ETEC infections are also a common cause of disease in young domestic animals [2]. ETEC exerts its pathogenic effects by producing heat-labile or heat-stable enterotoxins (LTs and STs, respectively).

There are two types of STs, STa and Stb, with different host specificity and protein structure [3]. Two kinds of STa molecules which vary only slightly have been identified; an STa subtype (STaI) produced by ETEC isolated from both humans and pigs which is 18 amino acids long and another (STaII) only isolated from strains infecting humans which is 19 amino acids in length [4,5]. Both enterotoxins have six cysteines which are known to form intra-chain disulfide bridges [5]. The importance of the disulfide bridges for the toxicity of STa is a subject of great interest because of its bearance on the design of safe vaccines for protection against ETEC diarrhea. Recently amino acid substitutions at the genetic [6] or synthetic protein level [7] have provided useful clues on the role of the cysteine residues.

Elaboration of protective antibodies against STa has the added requirement of having to make the protein, or its derivatives, immunogenic since the STa molecule itself is a very poor immunogen [8]. The problem of poor immunogenicity has been solved by coupling STa or its derivatives to larger carrier molecules so as to obtain effective antigens. Several proteins have been coupled chemically to STa for this purpose including BSA and the B-subunits of cholera toxin (CTB) and LT (LTB) [8,9]. The STa molecule has also been linked genetically to the A-subunit of LT (LTA) to give an LTA-STa hybrid protein which after associa-

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tion with unmodified LTB gave an LT holotoxin-like molecule exposing efficiently the STa antigenic epitopes [10,11]. However, all STa-derived hybrid proteins described so far bear unmodified and thus most likely toxic STa molecules and are therefore unsuitable for vaccination in humans. In an effort to create immunogenic hybrid proteins lacking STa toxicity we have fused genetically to the amino-end of CTB, a decapeptide whose primary sequence is identical to a region in native STa except for a mutation that replaces a cysteine residue by alanine. The decapeptide-CTB hybrid protein so obtained had no STa-associated toxicity but could give rise to antibodies cross-reacting with STa and was also efficiently recognized by neutralizing anti-STa antibodies.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and DNA manipulations*

The *E. coli* strain HB101 was used as transistent host for plasmid isolations. The *Vibrio cholerae* strain JS1569 (J.S. and J.H., unpublished) is a rifampicin-resistant derivative of strain CVD103 [12]. The *E. coli* strain S17-1 [13] was used for conjugal transfer of plasmids to strain JS1569. Plasmid pJS162 (J.S. and J.H., unpublished) encodes resistance to ampicillin. It is a derivative of the wide-host range plasmid pMMB68 [14] containing the CTB gene under the expression control of the IPTG-inducible *tac*P promoter. Plasmid pJS162 contains single *Sac*I and *Xma*I restriction sites at the junction between the leader peptide and the mature CTB-encoding DNA sequences. Isolation of plasmid DNA by the alkali-lysis method including centrifugation in CsCl/ethidium bromide gradients and DNA transformations were performed essentially as described by Maniatis et al. [15]. Conditions used for restriction and ligation of DNA were as recommended by the suppliers of the different enzymes (Boehringer Mannheim and New England Biolabs). The synthetic oligodeoxynucleotides encoding the STa-related decapeptide and adjacent amino acids (fig.1) were purchased as complementary individual strands from Dr Lena Samuelsson, Department of Immunology, Biomedical Centre, Uppsala, Sweden. These strands were paired by mixing equimolar amounts of each strand and incubating the mixture overnight at room temperature. The resulting double-stranded oligonucleotide contained single-stranded extensions compatible with *Sac*I at the 5'-end and with *Xma*I at the 3'-end and could therefore be ligated directly to *Sac*I-*Xma*I-restricted pJS162. Ligation was performed by incubating a 10-fold molar excess of oligonucleotide to plasmid DNA overnight at 4°C with T_4 DNA ligase. To the ligation mixture was then added in equimolar amounts with respect to the vector plasmid DNA a purified *Sac*I-*Xma*I fragment from plasmid pJ5162 containing the CTB gene, and the ligase reaction was then continued at 4°C for another 18 h. The ligated DNA was subsequently transformed into competent *E. coli* HB101 cells with selection for ampicillin resistance (100 μg/ml).

2.2. *Growth of strains*

Strains were kept at −70°C in LB medium [15] containing 15% glycerol until used. Cultures were grown overnight or until they reached the desired absorbance (600 nm) with continuous shaking at 37°C (*E. coli*) or at 30°C (*V. cholerae*) in liquid LB containing ampicillin (100 μg/ml) and/or rifampicin (50 μg/ml) as appropriate. Induction by isopropyl-β-D-thio-galactopyranoside (IPTG) of the expression of the *tac*P promoter was achieved either by addition of IPTG at the start of the culture (0.4 mM final concentration) or by first growing the strains to A_{600} 0.5 in the absence of the inducer and then adding IPTG to the desired concentration and continuing the growth for another 4 h before harvesting.

2.3. *ELISA assays*

After growth of strains in liquid LB as described in section 2.2, bacterial cells and culture supernatants were separated by centrifugation for 5 min in a microcentrifuge (Eppendorf). Cell pellets were resuspended in cold phosphate-buffered saline (pH 7.2) and disrupted by two 30-s sonic bursts (Branson sonifier). Detection of STa and CTB antigens in supernatants and cell sonicates was done by GM1-ELISA as described [9,16] using monoclonal antibodies directed against native STa or CTB, respectively [9,16].

2.4. *Immunoblots*

The decapeptide-CTB hybrid protein was purified from culture supernatants of *V. cholerae* strain JS1569 (pJS8) by single-step affinity chromatography on a lyso-GM1-spherosil® column essentially as described [17]. 10 μg of the purified protein or reference CTB were subjected to electrophoresis on 13.5% SDS-polyacrylamide gels in the absence of β-mercaptoethanol, and the separated proteins were then electrotransferred to nitrocellulose essentially as described in the Bio-Rad Catalogue for the Protean and Trans-Blot Units (Bio-Rad, Richmond, CA). Afterwards the nitrocellulose membrane was incubated with mouse anti-STa or anti-CTB monoclonal antibodies followed by incubation with anti-mouse IgG coupled to peroxidase (Jackson Biologicals). Immunoreactive protein bands were finally developed with the aid of a chloronaphthol substrate.

2.5. *Infant mouse toxicity assay*

The infant mouse toxicity assay [18] was used to assess any STa-related toxic activity of the decapeptide-CTB hybrid protein. Purified hybrid protein, or for comparison purified native STa [7,9], was injected intragastrically into 2–3-day-old mice who 3–4 h after injection were killed and examined for increased gut-to-body weight ratio as described [18].

2.6. *Immunizations and antibody assay*

Adult rabbits were immunized biweekly by two subcutaneous injections with 150 μg of purified decapeptide-CTB (equivalent to 12 μg of decapeptide) followed by a third injection with 1.2 mg hybrid protein (equivalent to 96 μg of decapeptide). In the first immunization the antigen was given together with complete Freunds adjuvant and in the subsequent ones with incomplete adjuvant.

Anti-STa reactive antibody titers in rabbit antisera were determined using a microtiter ELISA method [19] with plastic-attached synthetic STa as solid-phase antigen.

3. RESULTS

3.1. *Gene fusion of the STa-related decapeptide to CTB*

Plasmid pJS162 which contains single *Sac*I and *Xma*I restriction sites at the junction between the leader peptide and mature CTB was digested with the corresponding enzymes and then ligated to the synthetic oligodeoxynucleotide encoding the STa-homologous decapeptide (fig.1) via the *Sac*I and *Xma*I compatible ends. This resulted in plasmid pJS8 possessing a hybrid gene under the control of the *tac*P promoter that encodes a fusion protein in which the STa-related decapeptide, flanked by a few extra amino acids, is covalently linked to the amino-end of mature CTB (fig.1).

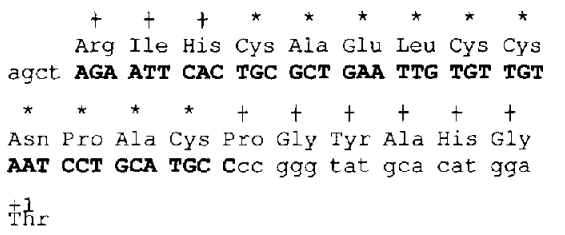

Fig.1. Fusion of STa-related decapeptide to CTB. A synthetic oligodeoxynucleotide (indicated by bold capital letters) with single-stranded extensions compatible to *Sac*I and *Xma*I restriction ends was inserted into the DNA region encoding the amino-end of the CTB in plasmid pJS162. Insertion of the synthetic oligonucleotide was performed so as to maintain the original reading frame in the CTB gene and resulted in a hybrid gene encoding a fusion protein containing a peptide extension comprising the STa-related peptide (indicated by asterisks) at the amino-end of CTB (first amino acid indicated by the +1 number). The amino acids signaled by (+) are amino acids encoded by the indicated oligonucleotide which are not considered to form part of the STa-related decapeptide. Upstream of the first amino acid (Arg), there is a leader peptide for CTB originating from a gene encoding LTB, a ribosome binding site (S/D) and the *tac*P promoter for expression of the hybrid gene (J.S. and J.H., unpublished).

3.2. *Expression of the STa-related decapeptide-CTB hybrid and its secretion by* V. cholerae

The STa-related decapeptide-CTB hybrid encoded by pJS8 was initially identified in transformed *E. coli* 101 grown in the presence of IPTG as described above. Plasmid pJS8 was subsequently transferred by conjugation to *V. cholerae* JS1569 using a helper *E. coli* strain (S17-1) as an intermediate host. The expression of the hybrid gene in *V. cholerae* JS1569 was then studied after culture with addition of different concentrations of IPTG during the logarithmic phase of growth and the cellular localization of the decapeptide-CTB protein was determined as well. The results in fig.2 show that the hybrid protein was produced in a clearly IPTG-dependent manner and was fully secreted to the extracellular milieu. This location of the decapeptide-CTB facilitated its purification by affinity chromatography.

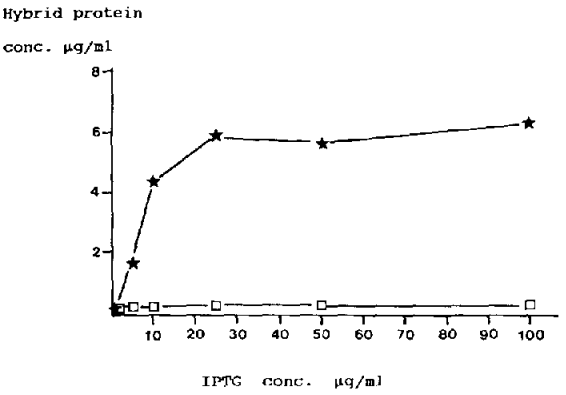

Fig.2. Induction of STa-related decapeptide-CTB hybrid gene expression by IPTG in *V. cholerae* JBK70 harbouring plasmid pJS8. Cells were grown in liquid medium to A_{600} 0.5 in the absence of inducer and then IPTG was added at the indicated concentrations and cultivation of bacteria continued for another 4 h before bacterial cells and culture supernatants were harvested as described in the text. Levels of hybrid protein (ordinate) were determined by GM1-ELISA using a specific monoclonal anti-STa antibody, ST 1:3. Purified STa-related decapeptide-CTB hybrid protein was used as a reference. Stars denote concentrations of hybrid protein supernatants and open squares cell-associated concentrations determined after disruption of the cells by ultrasonication.

The decapeptide-CTB protein exhibited no toxic activity tested in amounts of up to 10 μg in the infant mouse assay. Concurrently tested purified native STa gave positive infant mouse tests in amounts down to 1 ng, i.e. a 10000-fold lower dose.

3.3. *Immunogenicity of the STa-related decapeptide-CTB*

When the purified protein was used to immunize rabbits it gave rise to antibodies which cross-reacted with native STa. The anti-STa titer determined by ELISA using synthetic, plastic-coated STa as a solid phase antigen rose from an undetec-

table level in preimmunization serum to a titer of 1:2000 after three subcutaneous immunizations. This titer rise compared favourably with the anti-ST response attained in rabbits by immunization with a chemically derived hybrid protein carrying native STa [4].

3.4. *Immunoblot analyses of the STa-related decapeptide-CTB*

Purified STa-related decapeptide-CTB and control CTB were subjected to SDS-polyacrylamide electrophoresis in the absence of β-mercaptoethanol and the separated proteins reacted in parallel in immunoblot assays with anti-CTB and anti-STa. Protein samples were either boiled or left at room temperature in sample buffer before electrophoresis to facilitate analyses of the immunoreactive proteins in their pentameric and monomeric forms [11]. Reaction with anti-CTB revealed bands at the positions expected for the pentameric forms of the control CTB (fig.3, lane a) or STa-related decapeptide-CTB hybrid protein (fig.3, lane c). Incubation with anti-STa of the respective boiled and unboiled samples developed bands corresponding to the pentameric and monomeric forms of the decapeptide-CTB protein (fig.3, lanes g and h) and failed to react with either the pentameric or monomeric forms of the control CTB (fig.3, lanes e and f). The monomeric form of CTB did not react with anti-CTB (fig.3, lane b) whereas a minor reaction of the monomeric form of the decapeptide-CTB with this antibody was observed (fig.3, lane d).

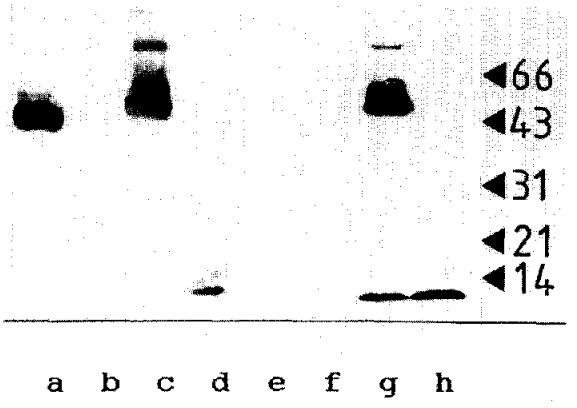

Fig.3. Immunoblotting analysis of STa-related decapeptide-CTB hybrid protein after SDS-polyacrylamide gel electrophoresis followed by electrotransfer of separated proteins onto nitrocellulose paper. Lanes show reaction patterns of unboiled purified 569B CTB (a), boiled CTB (b), unboiled STa-related decapeptide-CTB (c) and boiled STa-related decapeptide-CTB (d) after development with anti-cholera toxin monoclonal antibody Wi7:5. Lanes e–h show the reaction patterns obtained for the same proteins after development with anti-STa monoclonal antibody ST 1:3. Arrows refer to the migration positions of reference proteins with known molecular masses (indicated as kDa) run on the same gel.

4. DISCUSSION

The STa heat-stable enterotoxin of *E. coli* has six cysteine residues in its primary structure which are known to form three intra-chain disulfide bridges [5]. The cysteine residues are contained within a 15 amino acid stretch of the protein and this region is not only identical between the two different variants of STa molecules known (STa I and STa II) but it also shares extensive homology with the heat-stable enterotoxins from non-01 *V. cholerae* and *Yersinia enterocolitica* [20,21]. The multiple disulfide bridges in STa have been implicated in its toxicity [22] and they have thus become targets for manipulations with the aim to obtain safe vaccine candidates.

Amino acid replacements on STa using site-specific mutagenesis have provided important clues on the role of individual cysteines on the toxic properties of STa [6]. Similarly, chemical synthesis of cysteine-substituted non-toxic STa analogues able to bind anti-STa antibodies has recently furnished extra information on the significance of the disulfide bridges on some immunorelevant epitopes [7]. Substitutions or suppressions of the cysteine residues to eliminate the toxicity of STa have probably got to be done carefully so as not to alter the overall conformation of the protein risking to eliminate its protective epitopes. Here we have fused a synthetic DNA sequence encoding a decapeptide which is identical to a region contained in STa except for a replacement of one of the 5 cysteines in this region for an alanine residue. The decapeptide was fused to the CTB gene in plasmid pJS162, which is expressed from a recently developed *tac*P-directed overexpression system (J.S. and J.H., unpublished). The gene fusion resulted in the synthesis of a hybrid protein where the decapeptide was joined to the amino-end of CTB. The sequence of the decapep-

tide fused was chosen based on our recent finding of the anti-STa antibody-binding capacity of a non-toxic synthetic nonadecapeptide peptide which contains this amino acid replacement [7]. The sequence fused here is, however, shorter and comprises only 10 amino acids including four cysteines.

The decapeptide-CTB protein exhibited no toxicity in the infant mouse intragastric assay nor did the synthetic nonadecapeptide containing this sequence [7]. Furthermore, the STa-related decapeptide-CTB hybrid protein was able to efficiently bind anti-STa antibodies both in GM1-ELISA and in immunoblot analyses. The hybrid protein exhibited, in addition, a number of attributes characteristic of the CTB moiety such as the ability to pentamerize, to bind to the GM1 receptor and to be secreted by *V. cholerae* [23]. The latter two properties made possible purification of the decapeptide-CTB from *V. cholerae* culture supernatants by a single-step receptor-specific affinity chromatography procedure developed for the purification of CTB [17]. When the purified protein was used to immunize rabbits it gave rise to antibodies which cross-reacted with native STa. The anti-STa titers obtained compared favourably with the anti-STa titers attained using a chemically derived hybrid protein carrying STa [24]. This finding together with the lack of toxicity now warrants studies on the protective immunogenicity of the STa-related decapeptide-CTB protein against STa-associated ETEC diarrhea in experimental animals and, if promising results are obtained, also in humans. As mentioned above the decapeptide-CTB protein retained full capacity to bind to GM1 while reacting strongly with anti-STa antibodies. These properties allow the use of the hybrid protein as an immunoreagent for detection of STa by GM1-inhibition ELISA [23]. Indeed, evidence is now available indicating that not only the decapeptide-CTB hybrid can be used as a reagent to detect STa in culture supernatants of clinical isolates of enterotoxigenic *E. coli* but that this protein is superior to other genetically obtained ST-LT hybrids [10,11] in this test (J.S. and A.M.S., unpublished).

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