Antibody response against *Escherichia coli* heat-stable enterotoxin expressed as fusions to flagellin

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The heat-stable toxin (ST) produced by enterotoxigenic *Escherichia coli* strains causes diarrhoea by altering the fluid secretion in intestinal epithelial cells. Here, the effectiveness of a flagellin fusion protein of *Salmonella* containing a 19-amino-acid sequence derived from the ST sequence (FLA-ST) in generating antibodies capable of neutralizing the toxic activity of ST was evaluated. This fusion protein, and an alternative construction where two cysteine residues in the ST sequence were substituted by alanines (ST_{mt}), were delivered to the immune system by three distinct strategies: (i) orally, using an attenuated *Salmonella* strain expressing FLA-ST; (ii) intraperitoneally, by injection of purified FLA-ST; (iii) orally, using attenuated *Salmonella* carrying a eukaryotic expression plasmid (pCDNA3) with the gene encoding FLA-ST. The results showed that the flagellin system can be used as a carrier to generate ST-neutralizing antibodies. However, it should be mentioned that humoral immune response against ST was only obtained when the mutated ST sequence was employed. FLA-ST was found to be non-immunogenic when delivered via the oral route with attenuated *Salmonella* strains. However, a flagellin antibody response was obtained by immunizing mice with *Salmonella* carrying pCDNA3/FLA-ST_{mt}. Oral immunization with *Salmonella* carrying the eukaryotic expression plasmid (pCDNA3/FLA-ST_{mt}) seems to be a promising method to elicit an appropriate response against fusions to flagellin.

Keywords: ETEC, ST, attenuated *Salmonella*, flagellin fusions, mucosal vaccines

INTRODUCTION

The virulence of enterotoxigenic *Escherichia coli* (ETEC) strains, an important cause of diarrhoea in children in developing countries and travellers to these areas (Black, 1990, 1993), is directly related to the production of heat-labile (LT) and/or heat-stable (ST) toxins, which alter the hydrosaline balance of the intestinal mucosa. ST acts by stimulating guanylate cyclase in intestinal epithelial cells; the rise of cGMP ultimately results in extensive fluid secretion. The primary structure of ST has been determined and its full enterotoxigenic activity has been found to be expressed by a segment of 13 amino acid residues, which includes six cysteines linked by three disulfide bonds (Okamoto

et al., 1987; Shimonishi *et al.*, 1987; Yamasaki *et al.*, 1988; Yoshimura *et al.*, 1985).

Several lines of evidence have indicated that protection against ETEC diarrhoea involves anti-toxin and anti-bacterial components. ST is a non-immunogenic low-molecular-mass peptide (2 kDa); however, its conjugation to different carriers can lead to the induction of neutralizing antibodies. Recently, a number of efforts have been made to render ST immunogenic, including chemical coupling and genetic fusions to appropriate carrier proteins (Clements, 1990; Houghten *et al.*, 1984, 1985; Klipstein *et al.*, 1982, 1983; Sanchez *et al.*, 1986, 1988). In general, chemical coupling reduced ST-associated toxicity as a function of the cross-linking and maintained the immunological determinants of ST. Genetic fusions have, as an advantage, the possibility of being delivered by live attenuated bacterial strains to elicit antibody response on the mucosal surfaces. Fusion

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; OPD, o-phenylene-diamine dihydrochloride.

between ST and LT resulted in a non-toxic and immunogenic molecule, capable of eliciting neutralizing antibodies against ST (Clements, 1990). Moreover, sera and mucosal secretions from mice immunized orally with an attenuated *Salmonella* strain carrying this genetic fusion were able to neutralize the biological activity of native ST in the suckling mouse assay, but, surprisingly, in the absence of detectable ELISA antibody titres against ST (Cárdenas & Clements, 1993). The OmpC outer-membrane protein of *E. coli* has also been used as a carrier for ST, and antibody responses to ST and OmpC proteins were elicited in rabbits immunized subcutaneously with whole cells expressing the hybrid protein; however, these antibodies were not able to neutralize the ST toxic activity (Saarilahti *et al.*, 1989).

Attenuated *Salmonella* represents an attractive vector for the delivery of heterologous antigens to the immune system. *Salmonella* can be rendered avirulent, for example, by the inactivation of genes involved in the biosynthesis of aromatic compounds. A number of antigens have been expressed by these attenuated strains in attempts to construct bivalent vaccines. Heterologous epitopes have also been expressed as fusions to the *Salmonella* flagellin, a system developed to expose epitopes on the bacterial surface, as part of the flagellar filament (Newton *et al.*, 1989, 1991, 1995; Wu *et al.*, 1989). Attenuated bacterial strains have also been used as carriers for DNA delivery *in vivo*, because of their ability to deliver the antigen-encoding DNA specifically to antigen-presenting cells at inductive sites of immune response (Darji *et al.*, 1997; Paglia *et al.*, 1998; Sizemore *et al.*, 1995, 1997).

In this study, the flagellin system was used to present ST sequences to the immune system in order to obtain ST neutralizing antibodies. A sequence with 19 amino acids derived from the native ST toxin and an identical sequence except for two mutations that replace two cysteine residues by alanines were fused to flagellin, and delivered intraperitoneally as purified protein, or orally by attenuated *Salmonella* strains expressing the fusion protein or carrying a eukaryotic expression vector containing the fusion. The ability of these forms of presentation of the ST sequences to the immune system to elicit an immune response against ST is presented.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are shown in Table 1. Luria broth and Luria agar supplemented with 100 µg ampicillin ml⁻¹ were used for the growth of bacteria. Plasmid pLS408 is a pUC19 derivative carrying the *fliC(d)* gene (formerly called *H-1*) from *Salmonella muenchen* with a 48 bp *EcoRV* fragment deletion in the central hypervariable region (region IV) of the flagellin gene (Newton *et al.*, 1989). The eukaryotic expression vector CDNA3 (Invitrogen) was used for cloning the flagellin gene containing the ST_{mt} (mutant ST) fragment.

Synthetic oligonucleotides and insertions in the flagellin gene. Complementary oligonucleotide pairs corresponding to the ST wild-type (ST_{wt}) sequence (¹NSSNYCCELCCNPAC-

TGCY¹⁹) or to a mutant ST (ST_{mt}) (Cys⁷Ala and Cys¹⁸Ala) sequence were inserted in the central, hypervariable, region of the *fliC(d)* flagellin gene at the *EcoRV* site of vector pLS408. The oligonucleotide for the coding strand of ST_{wt} was 5'-ATCAATTCTTCTAAC TACTGCTGTGAACCTTTGTTG-TAATCCTGCCTGTACA GGATGTTACGTA-3' and of ST_{mt} was 5'-ATCAATTCTTCTAACTACTGCGCCGAAC-TTGTGTTGTAATCCTGCCTGTACAGGAGCCTACGT-A-3'. The insertions were sequenced using a 15-mer primer complementary to nucleotides located 26 bp upstream from the *EcoRV* insertion site in the *fliC* gene. The plasmids containing the ST inserts in the correct orientation were introduced into the *Salmonella enterica* serovar Typhimurium strain LB5000 and then transferred to *S. enterica* serovar Dublin strain SL5928.

Cloning the flagellin gene into the eukaryotic expression vector pCDNA3. For the construction of the eukaryotic expression vector pCDNA3 carrying the hybrid flagellin gene, a 1.8 kb *EcoRI*-*Sau3AI* fragment containing the flagellin gene with the ST_{mt} sequence was isolated from plasmid pLS408/ST_{mt}, and was inserted in the *EcoRI* and *Bam*HI sites of vector pCDNA3. The resulting plasmid, pCDNA3/FLA-ST_{mt}, was introduced into the *Salmonella typhimurium* strain LB5000 and transferred to *S. typhimurium* strain SL3261.

Detection of the hybrid flagellins. Flagellin, purified by acid cleavage as described by Ibrahim *et al.* (1985), and bacterial cell lysates were separated on 10% SDS-PAGE gels. The proteins were transferred to nitrocellulose filters (Hybond-C Extra; Amersham) at 1 A for 2 h using the buffer conditions described by Towbin *et al.* (1979). The membranes were blocked with 5% low-fat milk in PBS for 1 h at room temperature, and incubated for 1 h with rabbit antiserum against *Salmonella* flagellar antigen d (Difco). After washings with 0.05% Tween 20 in PBS (PBS-T), bound antibodies were reacted with goat anti-rabbit IgG conjugated with peroxidase (Sigma). After incubation for 1 h with the conjugate and washings in PBS-T, bound antibodies were detected using the ECL detection system (Amersham).

Immunizations. BALB/c mice, 6–8 weeks old, were immunized intraperitoneally with 10 µg purified flagellin added to 500 µg aluminium hydroxide as adjuvant on days 0, 21 and 35. Sera samples were obtained 28 d after the initial immunization and 7 d after the last booster. For oral immunization, 10¹⁰ c.f.u. of live *S. typhimurium* resuspended in 0.2 M NaHCO₃ were fed to 8–12-week-old mice on days 0, 21 and 35, and fecal and sera samples from immunized animals were obtained 7 d after the second and last doses. These groups of mice received one additional dose each on day 65, and fecal and sera samples were collected 7 d after final inoculation. Groups of BALB/c mice, 6–8 weeks old, were also immunized in the leg muscle with four doses of 100 µg CsCl-purified plasmid pCDNA3/FLA-ST_{mt}, diluted in 50 µl PBS, at 14 d intervals. Serum samples were obtained 7 d after the second, third and fourth doses.

Analyses of serum antibodies (ELISA). Microplates (Nunc) were coated with 100 ng purified flagellin per well and incubated at 4 °C overnight. The wells were washed three times with PBS-T and blocked with 5% low-fat milk for 1 h at 37 °C. Different dilutions of mice sera were added and the plates were incubated for 1 h at 37 °C. Washes with PBS-T were performed, anti-mouse IgG-peroxidase conjugate was added and incubation was continued for 1 h at 37 °C, prior to development with OPD (o-phenylenediamine dihydrochloride) and H₂O₂ as enzyme substrate. Detection of antibodies

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i> DH5 α	<i>lacZ</i>	Laboratory stock
<i>Escherichia coli</i> (ETEC) PB176	STa	Evans & Evans (1978)
<i>Escherichia coli</i> C600	K-12	Laboratory stock
<i>Salmonella typhimurium</i> LB500	r ⁻ m ⁺	Sanderson & Stocker (1987)
<i>Salmonella typhimurium</i> SL3261	<i>aroA</i>	Hoiseth & Stocker (1981)
<i>Salmonella dublin</i> SL5928	<i>aroA fliC::Tn10</i>	Newton <i>et al.</i> (1989)
<i>Salmonella dublin</i> SL5930	<i>aroA fliC::Tn10</i> , pLS408	Newton <i>et al.</i> (1989)
Plasmids		
pLS408	pUC19 derivative carrying gene <i>fliC(d)</i> from <i>Salmonella muenchen</i> with a 48 bp deletion and unique <i>EcoRV</i> site	Newton <i>et al.</i> (1989)
pLS408/ST _{wt}	pLS408 derivative with the ST portion coding insert	This study
pLS408/ST _{mt}	pLS408 derivative with the mutated ST portion coding insert	This study
pCDNA3	Eukaryotic expression vector	Invitrogen
pCDNA3/FLA-ST _{mt}	pCDNA3 derivative with the mutated ST portion coding insert	This study

against ST was performed as described by Svennerholm *et al.* (1986). Alternatively, microplates were coated with the ganglioside GM1 (Sigma) (1 mg ml⁻¹) diluted in PBS and incubated at 4 °C overnight. The wells were blocked with 0.1% BSA-PBS for 30 min at room temperature. ST coupled to the purified β subunit of cholera toxin (ST-CT_B) (a gift from Dr A.-M. Svennerholm, Göteborg University, Sweden), 500 ng ST ml⁻¹, diluted in PBS was added and incubation was continued for 1 h at room temperature. Different dilutions of mice antisera were added and plates were incubated for 1 h at 37 °C. Washes with PBS-T were performed, anti-mouse IgG conjugate was added and incubation was continued for 1 h at 37 °C, prior to development with OPD and H₂O₂ as enzyme substrate.

Inhibition ELISA. Sera from mice immunized with purified flagellin were pre-incubated with supernatants of overnight cultures, in Casamino acids/yeast extract (CAYE), of ETEC strain PB176 (STa), or of *E. coli* K-12 C600 as a negative control, and added to microtitre plate wells previously coated with GM1 (1 mg ml⁻¹) and ST-CT_B conjugate. Washes with PBS were performed and bound antibodies were reacted with goat anti-mouse IgG conjugated with peroxidase and developed with OPD and H₂O₂.

Neutralization of the ST toxic activity. The suckling mouse assay for ST was performed as described by Giannella (1976). Mice (1–3 d old) were inoculated intragastrically with 0.1 ml CAYE supernatant of ETEC strain PB176 previously incubated with sera from immunized mice, for 1 h at 37 °C. After 3 h inoculation, mice were killed, the intestines were removed and the ratios between the weights of the guts and of the remaining carcasses were calculated. Gut/carcass ratios of ≥ 0.082 were considered positive.

RESULTS

Expression of ST as fusions to *Salmonella* flagellin

Sequences encoding the native ST toxin were inserted in the central, hypervariable, region of the flagellin gene *fliC(d)* at the *EcoRV* site of plasmid pLS408. A mutant derivative of ST, where Cys⁷ and Cys¹⁸ were replaced by alanines, was also constructed by oligonucleotide in-

sertion in the same site of flagellin. This mutant version was employed in order to abolish, or decrease, a possible toxicity of the ST molecule. The plasmids were introduced in the attenuated *Salmonella dublin* strain SL5928, which does not express wild-type flagellin, in order to express and purify the hybrid flagellins. Hybrid proteins of the expected molecular mass for both constructions were detected in whole cells at approximately the same levels as the flagellin without insert (Fig. 1a). Cells carrying the hybrid flagellins were non-motile in semi-solid Luria medium. Because the complex structure of ST could be interfering with the assembly of the flagellum, bacterial agglutination assays were performed using anti-flagellin antibodies to verify the presence of the flagellum on the cell surface. This serum agglutinated strains expressing the hybrid flagellins (data not shown), indicating that the flagellins were present on the surface of the bacteria.

Immunogenicity of purified ST-flagellin fusions

To determine whether ST-flagellin could behave as a strong immunogen, the flagellins were purified from the *Salmonella* strains SL5930, SL5928/ST_{wt} and SL5928/ST_{mt} (Fig. 1b, c) and used to inoculate mice intraperitoneally. Sera from immunized mice showed high ELISA titres against flagellin (Table 2). Furthermore, high ELISA titres against ST were also detected in sera from mice immunized with FLA-ST_{mt} (purified flagellin containing the mutated ST sequences) (Table 2). The ability of antibodies generated against FLA-ST_{mt} to recognize the native ST toxin produced by ETEC strain PB176 was evaluated in inhibition ELISA tests. The binding of antibodies generated after immunization with the hybrid flagellin to solid-phase-bound ST-CT_B conjugate was partially inhibited after incubation in the presence of ST-positive culture supernatant (Fig. 2). Moreover, sera from mice immunized with the purified FLA-ST_{mt} were able to partially neutralize the bio-

Fig. 1. Detection and purification of the hybrid flagellins. (a) Immunoblot of whole-cell extracts of *S. dublin* expressing the wild-type flagellin (lane 1) and the FLA-ST_{wt} (lane 2) and FLA-ST_{mt} (lane 3) hybrid flagellins, reacted with an antiserum against flagellin. (b) Coomassie blue staining of 10% SDS-PAGE of the purified flagellins. (c) Immunoblot of purified flagellins using anti-flagellin antibodies. Molecular mass standards are indicated in kDa on the left.

Table 2. Mice IgG antibody responses against flagellin and ST

Mice were immunized intraperitoneally with flagellins or intramuscularly with pCDNA3/FLA-ST_{mt}. ELISA titres represent means of the reciprocal of the titres $\pm$ SD of pooled mice sera. Mice pre-immune sera were used as negative controls. An A_{492} value two times above the control sera was defined as positive.

Antigen	Serum antibody titre	
	Flagellin	ST
FLA-30	156 214 $\pm$ 5764	< 50
FLA-ST _{wt}	145 753 $\pm$ 3979	< 50
FLA-ST _{mt}	96 874 $\pm$ 2524	3937 $\pm$ 191
pCDNA3/FLA-ST _{mt}	1679 $\pm$ 216	< 50

Fig. 2. Inhibition ELISA. Sera from mice immunized with FLA-ST_{mt} were pre-incubated with the supernatant of an overnight culture of ETEC strain PB176 (STa) (shaded bars) or of *E. coli* K-12 C600 (striped bars), as negative control, and added to microtitre plate wells previously coated with GM1 (2 mg ml⁻¹) and incubated with ST-CT_B conjugate. The results are means of duplicates. Standard deviations were never greater than 10% above or below the mean.

Fig. 3. Neutralization of the ST toxic activity. The CAYE supernatant of ETEC strain PB176 (diluted 1:2) was incubated with sera from immunized mice (diluted 1:5), for 1 h at 37 °C, prior to inoculation in suckling mice. Gut/carcass ratios of ≥ 0.082 are considered positive. Values are means $\pm$ SD of four independent experiments performed with four or five mice in each group.

logical activity of the native ST in the suckling mice assay (Fig. 3).

Immunogenicity of FLA-ST_{mt} expressed in eukaryotic cells

We showed that mice immunized parenterally with the purified recombinant flagellin FLA-ST_{mt} developed neutralizing antibodies against ST. However, the desired mucosal immune response probably could not be achieved by this route of immunization and, as was reported in a previous work and confirmed here (data not shown), chimeric flagellins were not capable of eliciting an immune response when carried by *Salmonella* strains in oral immunization (Almeida *et al.*, 1999). The expression of the hybrid flagellin inside the antigen-presenting cells at gut-associated lymphoid tissues could be an alternative approach to improve the stimulation of specific antibody responses following oral administration. Therefore, an *S. typhimurium* strain carrying the flagellin gene with the mutated ST

Fig. 4. Immune response induced by FLA-ST expressed in eukaryotic cells. Western blot analyses using purified flagellin of sera from mice immunized intramuscularly with vector pCDNA3/FLA-ST_{mt} (lanes 1 and 2); sera from mice immunized orally with SL3261 carrying pCDNA3 (lanes 3 and 4); sera from mice immunized orally with SL3261 carrying pCDNA3/FLA-ST_{mt} (lanes 5 and 6).

insert under the control of a eukaryotic promoter in the pCDNA3 vector (pCDNA3/FLA-ST_{mt}) was constructed.

In order to verify the expression of the chimeric flagellin encoded by the plasmid pCDNA3/FLA-ST_{mt}, mice were initially immunized intramuscularly with the purified plasmid DNA. Antibodies against flagellin were detected in sera from mice immunized with four doses of DNA (Table 2). No antibody response was detected against ST. The anti-flagellin ELISA titre obtained was significantly lower than those obtained with intraperitoneal injections of the purified flagellin. This titre difference may explain the absence of detectable antibodies against ST in mice immunized with pCDNA3/FLA-ST_{mt} intramuscularly. However, these results indicated that the antigen was being expressed in eukaryotic cells, and that the construct could be employed with the attenuated live *Salmonella*.

Mice were then fed with live *S. typhimurium* carrying pCDNA3/FLA-ST_{mt} and the sera of these animals were tested for the presence of antibodies against flagellin. Because of the high anti-LPS antibodies generated by immunizations with intact bacterial cells, sera from immunized mice were tested in Western blot assays with purified flagellin to avoid mistaken interpretations given by the ELISA assays using flagellin preparations which could contain some LPS contaminants. An antibody response against flagellin was detected in sera from these immunized mice, as revealed by Western blotting (Fig. 4). These sera tested negative for reactivity against ST in ELISA (data not shown). This was expected given the low titres obtained for flagellin. However, these results indicate that this alternative route of oral delivery of flagellin seems to overcome the inherent lack of antigenicity of flagellin when presented by bacteria in oral immunizations.

DISCUSSION

ST enterotoxin is an important virulence factor of ETEC strains and has been frequently identified among ETEC strains isolated from children with diarrhoeal disease in Brazil (Gomes *et al.*, 1991; Guth & Trabulsi, 1985; Reis

et al., 1982). The small size and poor immunogenicity of the ST molecule have, however, hampered the development of a toxoid suitable for immunization against ETEC. In this study, sequences with 19 amino acids derived from the ST toxin were expressed as fusions to flagellin. Intraperitoneal immunizations with purified chimeric flagellins elicited neutralizing antibodies to ST, indicating that fusions to flagellin can render ST immunogenic. It is noteworthy that the humoral immune response against ST was obtained only when a mutated ST sequence (Cys⁷ and Cys¹⁸ replaced by alanines) was inserted in the flagellin. As previously reported, antigenicity and immunogenicity of ST in the genetic fusion with LT were obtained only when an appropriate proline-containing linker was inserted between the LT and ST sequences (Clements, 1990). The ST epitopes were probably masked by folding of the LT-ST fusion peptide in the absence of the linker. In agreement with this work, the conformation assumed by the flagellin containing wild-type ST may mask the ST epitopes and the disruption of the two ST disulfide bonds may expose important ST determinants. This result was not expected, since the main reason for using an altered version of ST in this work was to reduce its toxic effect, as was previously described for the genetic fusion of the ST-decapeptide (Cys⁶-Cys¹⁵) to the cholera toxin B-subunit, in which Cys⁷ was replaced by alanine, resulting in a non-toxic hybrid protein (Sanchez *et al.*, 1988).

The absence of an immune response against ST in mice immunized orally with *Salmonella* expressing FLA-ST reflects the non-immunogenic nature of flagellin through this route. Previous data have shown that serum and mucosal antibody responses against flagellin and the foreign epitope could be obtained following oral immunization with live attenuated *Salmonella* (Wu *et al.*, 1989). Recently, however, extensive work on the *Salmonella*/flagellin vaccine system presented evidence that flagellin does not represent an efficient carrier for heterologous epitopes by the oral route when delivered by live recombinant attenuated *Salmonella* strains (Almeida *et al.*, 1999).

An alternative approach employed in this study to overcome the non-immunogenic nature of flagellin through oral immunization was to obtain the expression of flagellin inside the mammalian cells. Attenuated *Salmonella* strains have been shown to be able to deliver plasmid DNA inside eukaryotic cells and expression of the antigen in the cytosol of macrophages and dendritic cells induced the stimulation of different arms of the immune system (Darji *et al.*, 1997; Sizemore *et al.*, 1997). Here, we showed that a systemic antibody response against flagellin could be elicited in mice orally immunized with attenuated *Salmonella* delivering the plasmid encoding the flagellin gene with the mutated ST to mammalian cells. Because the titres against flagellin were relatively low, we did not expect to detect an antibody response against the ST toxin. Nevertheless, this seems to be a promising method to elicit an appropriate response against fusions to flagellin. En-

hancement of the specific mucosal IgA immune response by expression of interleukin-5 by an attenuated *Salmonella* strain has been recently demonstrated (Whittle *et al.*, 1997). Moreover, co-administration of interleukin expression vectors with a DNA immunogen has been demonstrated to improve cellular and humoral immune responses (Chow *et al.*, 1997; Geissler *et al.*, 1997; Xiang & Ertl, 1995). The co-administration of vectors expressing interleukins and flagellin fusions delivered by attenuated *Salmonella* may improve the stimulation of a specific systemic and mucosal immune response against flagellin and heterologous antigen.

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