**Clostridium difficile** $\text{TxA}_{C314}$ and SLP-36kDa enhance the immune response toward a co-administered antigen

Paola Brun,¹ Melania Scarpa,¹ Alessia Grillo,¹ Giorgio Palù,¹ Carlo Mengoli,¹ Alfonso Zeconii,² Patrizia Spigaglia,³ Paola Mastrantonio³ and Ignazio Castagliuolo¹

¹Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Padua, Italy
²Department of Animal Pathology, Hygiene and Health, University of Milan, Milan, Italy
³Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

This study evaluated the *in vivo* adjuvant activity of two peptides derived from *Clostridium difficile*: a fragment of the receptor-binding domain of toxin A ($\text{TxA}_{C314}$) and a fragment of the 36 kDa surface-layer protein (SLP-36kDa) from strain C253. Their ability to affect the magnitude, distribution and polarization of the immune response against fibronectin-binding protein A (FnbpA), a protective vaccine antigen against *Staphylococcus aureus*, was evaluated using two different routes of immunization: intranasal and subcutaneous. It was shown that (i) the route of immunization affected the magnitude of the immune response; (ii) both peptides enhanced the production of circulating anti-FnbpA IgG and IgA; (iii) following mucosal immunization $\text{TxA}_{C314}$ was more effective than SLP-36kDa at inducing antibody in the gastrointestinal tract; (iv) the adjuvant influenced the Th1/Th2 balance; and (v) $\text{TxA}_{C314}$ was more effective than SLP-36kDa in inducing a cell-mediated response. These studies provide insight into the ability of different *C. difficile*-derived peptides to differentially affect and polarize the activity of the immune system and on their potential use as adjuvants in newly developed vaccines.

**INTRODUCTION**

Bacterium-derived products are the most powerful immunomodulatory molecules of the mucosal-associated immune system (Fallon & Alcami, 2006). Thus components of the bacterial cell wall, recognized through a family of surface and cytoplasmic receptors, i.e. Toll-like receptors and nucleotide-binding oligomerization domain isoforms, expressed in epithelial and immune cells modulate the development of adaptive immune responses against mucosal antigens (Cario, 2005; Nestle et al., 1998). Furthermore, bacterial toxins are among the strongest antigens and a few enterotoxins such as cholera toxin (CT) secreted by *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin (LT) are among the most powerful mucosal adjuvants identified to date (Aizpurua & Russell-Jones, 1988). Recent studies have reported that several *Clostridium difficile* proteins are immunodominant antigens, as they efficiently stimulate *in vivo* the immune system associated with the intestinal mucosa (Mukherjee et al., 2002). Thus significant levels of circulating antibodies against *C. difficile*-derived surface-layer proteins (SLPs), the predominant outer-surface component, toxin A (TcdA) and toxin B have been demonstrated in a large portion of the human population (Kyne et al., 2000). Indeed, *C. difficile*-derived proteins specifically interact with a variety of immune and non-immune cells in the intestinal mucosa. Thus TcdA binds specifically to epithelial cells and lamina propria phagocytes, inducing the release of a vast array of immunomodulatory cytokines (Castagliuolo & LaMont, 1999). We have also recently reported that a non-toxic fragment of the C-terminal domain of TcdA, $\text{TxA}_{C314}$, comprising a region of the receptor-binding domain, conserved the ability to bind to intestinal epithelial cells and to stimulate immune cells (Castagliuolo et al., 2004). $\text{TxA}_{C314}$ exerted a significant adjuvant activity following *in vivo* co-administration with weak peptide antigens, such as keyhole limpet haemocyanin and hen egg lysozyme.

**Abbreviations:** CT, cholera toxin; DC, dendritic cell; FnbpA, fibronectin-binding protein A; IFN-γ, gamma interferon; IL-2, interleukin-2; i.m., intramuscularly; i.n., intranasally; LT, heat-labile enterotoxin; s.c., subcutaneously; SLP, surface-layer protein; SLP-36kDa, fragment of the 36 kDa SLP; TcdA, toxin A.
(Castagliuolo et al., 2004). Recently, we and others (Cerquetti et al., 2002; Eveillard et al., 1993) have reported that SLPs from C. difficile bind to intestinal epithelial cells and induce the release of regulatory cytokines in dendritic cells (DCs) (Ausiello et al., 2006), suggesting that these proteins may also boost immune responses in vivo.

The goal of this study was to evaluate the ability of TxA<sub>C<sub>314</sub> and a fragment of the 36 kDa SLP (SLP-36kDa) to enhance and polarize the immune response toward a Th1- or Th2-type response. In particular, we evaluated the ability of TxA<sub>C<sub>314</sub> and SLP-36kDa to trigger a specific humoral, mucosal and cell-mediated response following mucosal or subcutaneous administration. As test antigen, we used a recombinant peptide comprising a surface adhesion factor, fibronectin-binding protein A (FnbpA), derived from a pathogenic Staphylococcus aureus strain that plays a key role in bacterial mucosal colonization (Lammers et al., 1999).

**METHODS**

**Expression and purification of recombinant TxA<sub>C<sub>314</sub>, SLP-36kDa and FnbpA.** TxA<sub>C<sub>314</sub>, SLP-36kDa and FnbpA were expressed in *E. coli* BL21(DE3). cDNAs encoding TxA<sub>C<sub>314</sub> from *C. difficile* strain VPI 10463 (ATCC) and FnbpA from *S. aureus* strain 17066 (Zeconci et al., 2005) were cloned in expression vector pGEX-2T as described previously (Castagliuolo et al., 2004, 2006). The protein SLP-36kDa from *C. difficile* C253 has been characterized previously (Cerquetti et al., 2000). In this study, the slpa gene region of 978 bp of p36 without the peptide signal was amplified using primers GST2 (5’-GCTGGATCCACTGGAACACAAGGT-3’) and GST7 (5’-TGTTATCATTTGCGAATTCAGTTTC-3’), incorporating a BamHI restriction site, and GST7 (5’-TGTTATCATTTGCGAATTCAGTTTC-3’), incorporating an EcoRI restriction site. The PCR fragment was cloned into the expression vector pGEX-6P-1. The recombinant proteins were purified by affinity chromatography using a glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech), according to standard procedures. The integrity, purity and concentrations of the recombinant proteins were determined by SDS-PAGE and Bradford (Bio-Rad) protein assays. Triton X-100 and bacterial LPS were removed by sequential single-step affinity chromatography using Calbiochrome Adsorbent Resin (Calbiochem) and Endotoxin Removing Gel (Pierce), respectively (Castagliuolo et al., 2004). The levels of endotoxin contamination in the recombinant protein preparations were determined to be less than 0.01 IU ml<sup>−1</sup> (*Limulus* amoebocyte assay; BioWhittaker), which corresponded to less than 0.1 μg LPS administered per mouse.

**Immunization protocols.** Male BALB/c mice (8–10 weeks old), purchased from Charles River Laboratories, Oderzo, Italy, were used in all experiments. Mice were housed under controlled temperature and humidity conditions and received standard pelleted chow and tap water *ad libitum*. All procedures involving animal studies were carried out following the guidelines recommended by the Institutional Animal Care and Use Committee of the University of Padova, Italy. Mice were injected either subcutaneously (s.c.) or intranasally (i.n.) with 10 μg purified peptide antigen (FnbpA) alone (negative-control mice) or with a peptide mixture consisting of equal amounts of recombinant TxA<sub>C<sub>314</sub> or SLP-36kDa as potential adjuvant together with FnbpA. In addition, a group of mice were injected either s.c. or i.n. with recombinant TxA<sub>C<sub>314</sub> or SLP-36kDa alone to assess the specificity of their effect. As a reference, 10 μg purified FnbpA mixed with Freund’s adjuvant was injected intramuscularly (i.m.).

A total of 8–12 mice were used for each experimental condition. Mice were bled 1 week prior to the first immunization and then randomly allocated to an experimental group. Three doses of vaccine were given at 2-week intervals: at time 0, and after 2 and 4 weeks. After a further 3 weeks, the animals were sacrificed. For i.n. administrations, 30 μl was transferred to the nasal cavity of non-anaesthetized mice through fine tips attached to a micropipette. For s.c. administration, a total of 30 μl was injected into the interscapular region of non-anaesthetized mice using a tuberculin syringe. For i.m. immunization, purified recombinant FnbpA was administered into the right tibialis anterior muscle supplemented with an equal volume of complete Freund’s adjuvant in a total volume of 30 μl. Control mice received equal volumes of sterile, endotoxin-free PBS, either i.n. or s.c.

**Body fluid collection.** Serum and intestinal washes were collected 3 weeks after the third immunization. Blood samples were collected in deeply anaesthetized animals by cardiac puncture. Blood was left at room temperature for 2 h, centrifuged and the serum was stored at −20 °C until used. The small intestine, from the duodenum to the ileo-caecal valve, was surgically removed and flushed with 3 ml ice-cold PBS containing a protease inhibitor mixture (50 mM EDTA, 120 μg aprotinin ml<sup>−1</sup>, 150 μg tosyl-l-lysine chloromethyl ketone ml<sup>−1</sup>, 20 μg pepstatin A ml<sup>−1</sup>, 10 μg leupeptin ml<sup>−1</sup>, 10 μg antipain ml<sup>−1</sup> and 10 μg chymostatin ml<sup>−1</sup>; Roche). Washes were centrifuged (10 000 g for 10 min) to remove faecal matter and mucus. The clear supernatants were collected and stored at −20 °C.

**Detection of specific antibodies by ELISA.** To determine the level of specific anti-*S. aureus* FnbpA in the serum and intestinal washes, standard indirect ELISAs were performed as described previously using purified recombinant *S. aureus* FnbpA as the capture antigen. To quantify anti-FnbpA antibodies, for each plate a reference curve was determined by coating the microtitre plate wells with known amounts of purified mouse IgG and IgA (ICN). The concentration of specific anti-FnbpA antibodies was determined by subtracting the mean value obtained in non-immune animals (background) from values obtained in each experimental (immune) mouse (Castagliuolo et al., 2004).

**Purification of spleen mononuclear cells and assessment of cell-mediated immune responses.** Spleens were removed aseptically 49 days after the primary immunization, and spleen mononuclear cells were purified and cultured as described previously (Castagliuolo et al., 2004). Cells from each animal were seeded at a concentration of 10<sup>6</sup> ml<sup>−1</sup> and cultured in medium alone or in the presence of purified FnbpA (10 μg ml<sup>−1</sup>) in a humidified 5% CO<sub>2</sub> incubator at 37 °C. As a positive control, cells were exposed to phytohaemagglutinin at 5 μg ml<sup>−1</sup>. To measure cytokine release, after 5 days, cultures were harvested by centrifugation and the supernatants were collected, fractionated and stored at −80 °C. The levels of interleukin-2 (IL-2) and gamma interferon (IFN-γ) were measured using commercially available ELISAs (Biosource), running each sample in duplicate. To assess lymphocyte proliferation after 96 h, [<sup>3</sup>H]thymidine (1 μCi (37 kBq) per well) was added to each well and cells were cultured for an additional 24 h before harvesting. Cells were then collected by centrifugation and washed twice with ice-cold PBS, and [<sup>3</sup>H]thymidine incorporation was determined using a β-scintillation counter. Splenocyte proliferation was expressed as the proliferation index, as described previously (Castagliuolo et al., 2004).

**Statistical analysis.** Results were expressed as means ± SEM. Statistical analysis was performed using analysis of variance and Bonferroni’s test. Statistical significance was considered for *P* < 0.05.
RESULTS AND DISCUSSION

**TxAC314 and SLP-36kDa enhance the production of anti-FnbpA-specific serum IgG and IgA**

Mucosal and subcutaneously administered vaccines are extremely appealing, as they can ideally trigger systemic and mucosal responses (Holmgren et al., 2003). However, the strength of the specific immune response elicited is generally disappointing due to inadequate numbers of DCs at the site of antigen administration and the poor efficacy of the adjuvant present in the vaccine in activating DCs and priming T-lymphocyte responses. Thus strategies to augment the immunogenicity of mucosal-delivered vaccines are intended mainly to identify substances able to induce DC recruitment, activation and maturation (McKenzie et al., 2004). It has recently been reported that *C. difficile*-derived SLPs are able to modulate DC activity (Ausiello et al., 2006), and in preliminary in vitro studies, we have observed that TxAC314 adjuvant activity in vivo depends on DC activation; therefore, we assessed the ability of recombinant peptides to modulate the immune response towards a surface antigen of *S. aureus* able to confer protective immunity against tissue colonization (Mamo et al., 1994). As shown in Fig. 1, following s.c. or i.n. immunization of mice with TxAC314 or SLP-36kDa co-administered with recombinant *S. aureus* FnbpA, we observed a significant increase in specific anti-FnbpA IgG in all of the animals immunized compared with administration of the peptide antigen alone. Indeed, TxAC314 and SLP-36kDa were equally effective at enhancing specific IgG production following i.n. or s.c. administration, whereas TxAC314 was more effective than SLP-36kDa in enhancing the production of FnbpA-specific serum IgA. In addition, in accordance with previous reports, s.c. administration was more effective than i.n. administration at triggering specific systemic antibody responses (De Magistris, 2006). Serum antibodies reacted with specific recombinant proteins as well as with native proteins obtained from lysates of reference *S. aureus* strains, as determined by ELISA and Western blotting (data not shown). In addition, s.c. or i.n. administration of TxAC314 or SLP-36kDa alone did not affect the level of specific anti-FnbpA IgG or IgA (data not shown).

**TxAC314 and SLP-36kDa induce a mixed immune response**

As polarization of the immune response plays a key role in protecting the host against specific pathogens, to characterize further the adjuvant activity of TxAC314 and SLP-36kDa we determined the serum levels of anti-FnbpA-specific IgG1 and IgG2a, following either i.n. or s.c. vaccine administration (Holmgren et al., 2003; De Magistris, 2006), the i.n. route was more effective than the s.c. route of administration in stimulating mucosal anti-FnbpA IgA. Furthermore, TxAC314 was slightly more effective than SLP-36kDa at enhancing mucosal anti-FnbpA IgA production following i.n. administration.
administration. As shown in Fig. 3, both immunization protocols with a peptide antigen in combination with TxAC314 or SLP-36kDa induced a mixed Th1/Th2-type immune response, as indicated by the production of both IgG subclasses (IgG1 and IgG2a). Mice immunized with FnbpA plus TxAC314 or SLP-36kDa via the s.c. route showed higher FnbpA-specific IgG2a levels than following vaccine administration through the i.n. route, supporting previous results indicating that the s.c. route is more effective at polarizing the immune response towards a Th1 response (Uddowla et al., 2007).

**TxAC314 is more effective at inducing antigen-specific cell-mediated responses**

As shown in Fig. 4, spleen lymphocytes obtained from mice immunized s.c. or i.n. using only recombinant FnbpA exhibited a modest proliferative response and negligible release of IFN-γ and IL-2 following in vitro challenge with recombinant FnbpA or *S. aureus* lysate (data not shown). However, spleen cells isolated from mice immunized with FnbpA in the presence of TxAC314 or SLP-36kDa and challenged in vitro with the peptide antigen released significant amounts of IL-2 and IFN-γ and showed a significant increase in the proliferative response (Fig. 4). In addition, lymphocyte proliferation and cytokine release were significantly more pronounced in cells purified from mice immunized with FnbpA in the presence of TxAC314 and challenged in vitro with FnbpA than in cells obtained from mice immunized with SLP-36kDa and FnbpA (Fig. 4), either i.n. or s.c.

Thus co-administration of either TxAC314 or SLP-36kDa with a peptide antigen induced a strong humoral and cell-mediated immune response. The profile of antigen-specific IgG subclasses and of cytokine released from immune splenocytes challenged in vitro with *S. aureus* FnbpA is compatible with the induction of a mixed Th1/Th2-type immune response for both *C. difficile*-derived proteins. However, TxAC314 induced a stronger cell-mediated response and higher levels of IgG2a, indicating a more pronounced Th1-type branch. This complex and less well-defined profile of the immune response is not unprecedented: following immunization, Th1- and Th2-type responses can be observed at different stages of the
immune response and depend on specific antigen/adjuvant combinations as well as on the route of vaccine administration (Flegel et al., 1991; Nakagawa et al., 1996). The molecular mechanisms leading to a more prominent production of Th1- or Th2-type cytokines and different IgG isotypes are presently not completely understood; however, we can speculate that both TxAC314 and SLP-36kDa can activate different intracellular signal cascades, which eventually trigger the release of different cytokines from immune and non-immune cells (Ausiello et al., 2006; Moser, 2001; Warny & Kelly, 1999). Cytokines present in the microenvironment at the time of priming, as well as the relative expression of co-stimulatory molecules, are key elements driving the CD4+ T-cell response towards a particular Th profile and therefore boost the development of an immune response or favour tolerance (Jones et al., 2001; Moser, 2001). In addition, as DCs, with pivotal roles in the initiation and subsequent development of immune responses, are a heterogeneous family of cells strongly influenced by the surrounding microenvironment, it is possible that DCs positioned in the nasal mucosa and subcutaneous tissues respond differently to the same molecules (Svensson & Kaye, 2006; Svensson et al., 2004).

A common feature of the most powerful mucosal adjuvants, such as CT, E. coli LT and plant lectins, is a carbohydrate-recognition domain able to bind simultaneously to several specific membrane receptors, inducing their clustering (Rappuoli & Pizza, 2000). Indeed, nontoxic CT and LT mutants retaining the ability to bind to mucosal epithelial cells maintain their adjuvant activity (Pizza et al., 2001). Both peptides used in this study exhibit in principle the ability to bind and cluster multiple
receptor molecules on the cell membrane. Indeed, SLP monomers can auto-assemble in regular supramolecular structures, whereas TxA<sub>2</sub>C<sub>314</sub> comprises a region of the receptor-binding domain of TcdA that shows a lectin-like structure (Castagliuolo et al., 2004; Heyman et al., 2007). Therefore, it is possible that, following binding to cell membranes, these peptides can induce receptor clustering that triggers the development of specific intracellular signalling cascades, activating immune cells and enhancing the immune response.

In summary, we have shown that two immunodominant peptides derived from <i>C. difficile</i> – a recombinant nontoxic region of the C-terminal domain of TcdA and a region of the 36 kDa SLP – demonstrate robust adjuvant properties when co-administered with a relevant peptide antigen via the mucosal (i.n.) or s.c. route. In addition, the profile of antigen-specific IgG subclasses and of cytokine release from immune splenocytes challenged <i>in vitro</i> with the test antigen used in this study is compatible with a different ability to polarize the immune response.

REFERENCES


