Nasal mucosal immunogenicity for the horse of a SeM peptide of *Streptococcus equi* genetically coupled to cholera toxin

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Abstract

The intranasal immunogenicity of cholera toxin (CT) genetically coupled to peptide sequence aa236-334 (F3) of the SeM protein of *Streptococcus equi* was studied in five young adult Welsh ponies. All ponies made rapid CTB- and SeMF3-specific serum antibody responses following the first immunization. Specific nasal IgA responses were detected in two ponies 14 days after the first immunization, in another two 14 days after a second immunization on day 14, and in all ponies 28 days after a third immunization on day 42. SeMF3-specific antibody responses in sera and nasal washes were dominated by IgGb and IgA, respectively, and remained elevated for at least 140 days. Strong serum IgGa and IgG(T) responses were also observed. These antibody responses were qualitatively similar to those induced during recovery from equine strangles. Antibody responses in mucosal secretions were boosted in some ponies by immunizations subsequent to the first immunization, but antibodies in serum were never boosted. In vitro survival of *S. equi* was significantly reduced by SeMF3-specific antibodies in sera obtained 14 days after the second immunization but survival increased in sera collected following subsequent immunizations, possibly due to absence of synthesis of high affinity antibodies. Finally, the susceptibility of all immunized ponies to commingling challenge by *S. equi* indicated either that SeMF3 lacks protective epitopes or that the antibodies induced by the chimera were not at effective levels. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Mucosal surfaces constitute the largest vertebrate organ system and are a primary barrier to entry of microbial pathogens. Besides very effective mechanical and physicochemical clearance mechanisms, these mucosal surfaces are guarded by a local immune system. Stimulation of these local protective immune responses requires local induction because of compartmentalization of the systemic and secretory immune systems. Since parenteral immunization does not usually result in satisfactory mucosal responses, much recent study has been directed at developing efficient means of stimulating both immune compartments by local application of vaccines [1].

Cholera toxin (CT) is a potent mucosal immunogen and adjuvant [2–4]. Its toxigenic sub-unit A (CTA) activates the adenylate cyclase system and the pentameric B (CTB) binds cell membrane Gb1 gangliosides thus enhancing its uptake by mucosal associated lymphoid tissue. This property has been utilized in the mucosal delivery of CT mixed with or genetically or chemically coupled to foreign antigens to induce mucosal and systemic immune responses to protein antigens [5–8]. CTB including small amounts of CT has also been used to induce mucosal and systemic antibody response in the horse to equine influenza virus hemagglutinin protein [9], and to inactivated equine influenza virus [10]. The former study [9] revealed stimulation of equine influenza virus-specific IgGa and IgGb, moderate IgG(T) responses in serum; and strong specific nasal mucosal IgA responses. These antibody responses were similar to those induced during recovery from equine influenza virus infection. The latter study [10] showed that neutralizing antibodies were induced in the nasal mucosa and that IgA was the dominant isotype; specific antibodies induced in serum were predominantly IgG. The aim of the present study was to investigate the nasal mucosal immunogenicity in the horse of CT genetically coupled to an immunogenic polypeptide of SeM, an anti-phagocytic M-like protein of *Streptococcus equi* [11]. *S. equi* causes equine strangles, a highly contagious upper respiratory tract infection characterized by pharyngitis and...
abscessation of lymph nodes of the head and neck [12]. Its major surface protein SeM stimulates strong nasopharyngeal mucosal and systemic antibody convalescent responses [13] which are believed to contribute to resistance to re-infection.

2. Materials and methods

2.1. Experimental animals

Healthy Welsh ponies, 2–3-year-old, raised in isolation for 30 days prior to exposure to S. equi or its antigen were used for immunizations.

2.2. Preparation of CT-SeMF3 chimera

Construction of the recombinant plasmid for expression of CT-SeMF3 chimeric protein is summarized in Fig. 1. Plasmid pCTΔA1 [5] containing ctA2 and the entire ctB gene was modified to facilitate further purification of CTB-A2. The plasmid was designated pCTΔA1h. The fragment of sSm encoding the immunogenic peptide aa236–334 (SeMF3) [14] was amplified by PCR and inserted between the thrombin cleavage site sequences between the pelB His-Tag fragment of pET15-b (Novagen) containing the CT-SeMF3 chimera by inserting the NcoI-BamHI fragment of pET15-b (Novagen) containing His-Tag and thrombin cleavage site sequences between the pelB signal sequence and A2 coding sequence. The plasmid was designated pCTΔA1h. The fragment of SeM encoding the immunogenic peptide aa236–334 (SeMF3) [14] was amplified by PCR and inserted between the BamHI and XhoI sites of pCTΔA1h. The recombinant plasmid was designated pCTΔSeMF3. Plasmids were transformed into E. coli strain DH5α. The plasmid was designated pCTΔSeMF3. The fragment of SeM encoding the immunogenic peptide aa236–334 (SeMF3) [14] was amplified by PCR and inserted between the BamHI and XhoI sites of pCTΔA1h. The recombinant plasmid was designated pCTΔSeMF3.

2.3. Immunizations

Five yearling ponies (#87, 90, 93, 94 and 95) were immunized intranasally on days 0, 14, 42, 70 and 126 with CT-SeMF3 in a volume of 4 ml of phosphate buffered saline, pH7.4 (PBS) using a Devilbiss 251 nasal atomizer. The first dose was 500 μg and subsequent doses were 300 μg. Serum and nasal wash samples were collected on days 0, 14, 28, 42, 70, 126, and 140. A control group of three ponies (#19, 20 and 71) was immunized intranasally on days 0, 21 and 56 with CTB-A2 alone, and serum and nasal wash samples collected at days 0, 21, 56, and 70. A control group consisting of ponies inoculated intranasally with SeM alone was not included because a previous study [15] had shown that SeMF3 alone elicited only a short-lived mucosal IgA and no systemic response.

2.4. Collection and processing of samples

Blood was collected by jugular venipuncture and serum harvested and stored at −20°C until analyzed. Nasal wash samples were obtained and processed as described previously [13]. Nasal washes and sera were adsorbed at 37°C for 30 min with a suspension of heat-killed (56°C for 30 min) S. zooepidemicus W60 to remove cross-reactive antibodies [13].

2.5. Challenge study

Two horses were infected by intranasal inoculation of 1.5 × 10^9 colony forming units (CFU) of an overnight broth culture of S. equi strain CF32. Both horses became febrile and depressed 5 days after inoculation and were then commingled on day 140 with the horses immunized with CT-SeMF3 or CTB-A2. Following commingling, the animals were examined each day and rectal temperatures, nasal discharge, lymphadenopathy or other clinical signs of strangles recorded.

2.6. Assay for CTB- and SeMF3-specific IgA and IgG sub-isotypes

An indirect enzyme linked immunosorbent assay (ELISA) utilizing SeMF3 and CTB, and monoclonal antibodies (MAbs) specific for horse IgGa (CVS48), IgGb (CVS39), IgG(T) (CVS38) and IgA (BV52) [13], was performed to determine levels of CTB and SeMF3-specific IgA and IgG sub-isotypes in serum and nasal washes. MAbs were used at titers with similar activities. For this, the individual purified immunoglobulin subtypes were coated at a concentration of 0.3 μg/ml in 0.1 M carbonate bicarbonate buffer, pH 9.2. Each MAb was titrated against its respective antigen to obtain an optimum dilution determined from the absorbance (ELISA OD) at the same point on the slopes of the titration curves. The optimum dilutions were: CVS48, 1/125; CVS38, 1/62.5; CVS39, 1/62.5; and BV52, 1/250.
SeMF3 (2 μg/ml) and CTB (1 μg/ml) were diluted in 0.1 M carbonate bicarbonate buffer, pH 9.2 and dispensed at the rate of 100 μl per well of flexible 96-well flat bottom polystyrene microtiter ELISA plates (Falcon, Becton Dickinson, Oxnard, CA). The plates were incubated overnight at 4°C and then washed three times with PBS containing 0.05% Tween 20 (PBS-T). The uncoated sites of the wells were blocked by adding 100 μl of 2% non-fat dry milk powder in PBS-T to each well and incubating for 1 h at 37°C. After washing, 100 μl of serum (dilution 1/100) and nasal washes (dilution 1/10) were added to duplicate wells. Positive primary antibody control wells consisted of positive serum and nasal wash samples obtained from a convalescent horse. After incubation at 37°C for 1 h and washing, 100 μl per well of the MAbs at previously determined dilutions were added and incubated again for 1 h at 37°C. 100 μl per well of 1/1000 diluted HRP conjugated rabbit anti-mouse IgG (Fc specific) (Sigma, USA) was added and incubated at 37°C for 1 h. After a final wash, 100 μl per well of substrate consisting of 0.07% orthophenylenediamine and 0.05% hydrogen peroxide in citric acid–phosphate buffer pH 5.0, was added to each well. The reaction was stopped after 5-10 min by adding 50 μl per well of 2 M sulfuric acid and the absorbance read at 490 nm in an automated microplate reader (EL310 Microplate Autoreader, Bio-Tek Inc, Winooski, VT). A positive result was scored as a value greater than or equal to 2 after calculating the sample to background ratio.

2.7. Assay for binding of CT-SeMF3 to G.M1 ganglioside

An indirect ELISA utilizing monosialoganglioside-GM1 (Sigma, USA) as coating antigen, CT-SeMF3, and antibodies specific for CTB and SeMF3, was performed. G.M1 ganglioside (1 μg/ml) was diluted in 0.1 M carbonate bicarbonate buffer, pH 9.2 and dispensed at the rate of 100 μl per well of a flexible 96-well flat bottom polystyrene microtiter ELISA plate. The plate was incubated overnight at 4°C and then washed three times with PBS-T. After blocking with 2% non-fat dry milk powder in PBS-T, 1 μg of CT-SeMF3 in 100 μl of PBS-T was added to all wells. After incubation at 37°C for 1 h and washing, 100 μl per well of each of a 1/100 dilution of rabbit anti-CT (Sigma, USA), equine anti-SeMF3, and normal rabbit and pony sera were added to duplicate wells and serially diluted 10-fold. Following another incubation for 1 h at 37°C, 100 μl per well horse radish peroxidase (HRP) conjugated protein G diluted 1/4000 was added to the entire plate and incubated at 37°C for 1 h. The plate was developed and read as described above.

2.8. Indirect bactericidal activity

Opsonophagocytic antibodies for S. equi in sera of ponies immunized with CT-SeMF3 were measured in an indirect bactericidal assay as described previously [13]. Briefly, 15 μl of an appropriately diluted suspension of an overnight culture of S. equi CF32 was incubated with 75 μl serum for 30 min at 37°C. Fresh heparinized blood (500 μl) from a donor pony raised in isolation with no known exposure to S. equi was added as a source of complement and phagocytes. After 3 h rotation at 37°C, duplicate pour plates were prepared by adding 150 μl of blood bacteria mixture to 15 ml tryptic soy agar containing 2% horse blood and incubated for 18 h. The number of colonies on each plate was then counted.

2.9. Statistical analysis

The mean count of surviving bacteria (CFU) for a serum sample at a particular date was compared with the corresponding mean value at day 0 using the Student’s t-test. P values of less than 0.05 were considered significant.

3. Results

3.1. Preparation and analysis of CT-SeMF3

Insertion of the His-Tag sequence upstream of seMF3 in pCT-SeMF3 facilitated isolation of pure CT-SeMF3 chimera by one-step chromatography of clarified cell lysate. SDS-PAGE of purified chimera revealed two bands with molecular weights corresponding to the B sub-unit of CT and the CT-SeMF3 fusion. Probing of the bands with antisera against CT and SeM confirmed their identity (Fig. 2). Because only the CT-SeMF3 fusion contains the His-Tag sequence, these results verified correct assembly of the chimera. Size exclusion chromatography through Toyopearl HW-55S resin (Rohm and Haas, Philadelphia) of the purified protein, monitored by ELISA developed with CT- and SEM-specific antisera, revealed two peaks (Fig. 3). The estimated molecular weights of the peaks corresponded with those calculated for the monomeric (82,600) and dimeric
(165,200) forms of the chimera. The presence of dimers is explained by a high calculated probability that molecules of SeMF3 form coiled-coil structures [15]. The chimeric protein also showed affinity for Ga11 ganglioside (data not shown).

3.2. CT-SeMF3 binding to GM1 ganglioside

Strong binding of CT-SeMF3 to Ga11 ganglioside was detected indicating the presence of a functional CTB pentamer (results not shown). Both rabbit CT specific and equine SeMF3-specific antisera showed reactivity up to dilutions of 1/10,000 whereas normal rabbit and pony sera were unreactive.

3.3. Serum antibody responses of ponies immunized with CT-SeMF3

All ponies immunized with CT-SeMF3 made CTB and SeMF3-specific serum antibody responses after the first immunization (Figs. 4 and 5). Although IgGb dominated, strong specific IgGa and IgG(T), and slight IgA responses were also observed in this group of ponies. Subsequent immunizations did not boost declining antibody levels.

3.4. Serum antibody responses of ponies immunized with CTA2

Pony #71 made strong CTB-specific IgGa, IgGb and IgG(T) responses following primary immunization (results not shown). Subsequent immunizations did not boost its declining antibody levels. Strong CTB-specific IgGa, IgGb and IgG(T) in ponies #19 and 20 did not appear until after the third immunization (results not shown). SeMF3-specific serum antibody responses were not observed in this group of ponies.

3.5. Nasal wash antibody responses of ponies immunized with CT-SeMF3

Nasal wash isotype responses of all ponies and some important individual IgG sub-isotype responses are shown (Figs. 4–6). Only pony #94 made good CTB and SeMF3-specific IgGb responses after the second immunization which were maintained until week 10 and declined thereafter (Fig. 6). Pony #94 also made a CTB-specific but no SeMF3-specific IgGa response after the second immunization (Fig. 6). Pony #87 made a strong SeMF3-specific but no CTB-specific IgGb response after the second immunization. The SeMF3-specific response was maintained until week 10 and declined afterwards (Fig. 6). The other
ponies made no specific IgGa and IgGb, and CTB-specific IgG(T) was never detected in nasal washes of any pony. Pony #94 made a strong SeMF3-specific IgG(T) response after the second immunization which was maintained until week 10 and declined thereafter (Fig. 6).

Since all ponies made CTB and SeMF3-specific IgA responses (Figs. 4 and 5), this isotype response is not shown for each pony. However, the time of onset of specific IgA responses differed among the ponies. Ponies #93 and 95 made good CTB and SeMF3-specific IgA responses following the first immunization whereas ponies #87 and 94 did not respond until after the second immunization. Pony #90 did not respond until after the third (CTB-specific IgA) and fourth (SeMF3-specific IgA) immunizations.

3.6. Nasal mucosal antibody responses of ponies immunized with CTB

Pony #71 made good IgGa, and strong IgGb and IgA responses to CTB following the first immunization (data not shown). Pony #20 made a strong CTB-specific IgA response following the third immunization. CTB-specific antibody was not detected in nasal washes of pony #19. No SeMF3-specific antibody responses were observed in this group of ponies.

3.7. Bactericidal (opsonophagocytic) antibody

Significant \((P < 0.05)\) opsonophagocytic (bactericidal) activity of serum antibodies was detected in sera from all ponies collected on week 4 following the second immunization (Table 1). Bactericidal activity in ponies #87, 90 and 94 was detected at week 10 following the third immunization, in pony #94 at week 18 following the fourth immunization, and in ponies #87 and 94 at week 20 following the fifth immunization. Sera from pony #94 showed significant bactericidal activity from week 2 to the end of the observation period.

3.8. Resistance to challenge by commingling exposure

All immunized ponies developed signs of strangles within 12 days of commingling exposure.
4. Discussion

The streptococcal sequence chosen for this study was from the central region of ScM which had previously been shown to contain a number of linear epitopes reactive with antibodies in convalescent sera and nasal washes of almost all horses [14]. Additionally, the sequence was predicted to have a structure which would favor correct self assembly of the chimera. Immunoblot analysis, size exclusion chromatography and affinity to GM1 ganglioside confirmed that self assembly had occurred (Figs. 2 and 3). Interestingly, CTB and SeMF3-A2 derived from both the periplasm and cytoplasm assembled efficiently and were easily isolated in highly purified form by one-step affinity chromatography on resin bound nickel. These observations indicate that production of purified chimera is feasible and economic.

The results demonstrate that intranasal administration of CT-SeMF3 effectively delivers SeMF3 to mucosal sites and elicits specific and persistent antibody responses in serum and in nasal secretions of the horse. These antibody responses are qualitatively and quantitatively similar to those induced during recovery from equine strangles in that antibody responses are dominated by IgG in serum and by IgA in nasal secretions along with strong specific serum IgGa and IgG(T) responses [13].

CTB and SeMF3-specific mucosal IgA paralleled serum IgG responses in ponies #93 and #95 but not in ponies #87, 90 and #94. Mucosal responses of the latter were delayed and required two or three intranasal administrations of CT-SeMF3. This delay in IgA production is unexplained but has been observed in an earlier study in ponies in which intranasally administered inactivated equine influenza virus with CTB and holotoxin elicited serum CTB-specific IgG responses after the first administration, whereas CTB-specific nasal IgA did not appear until after the second administration [10]. A lag in stimulation of local IgA has also been reported in mice [5].

Stimulation of SeMF3-specific antibody responses in serum and nasal secretions of all ponies was achieved following the first and third intranasal administrations of CT-SeMF3, respectively. However, SeMF3-specific antibody levels were not boosted in serum by a second and third administrations. A fourth and fifth administration carried out to boost declining serum antibody levels also had no noticeable effect suggesting that CTB and SeMF3-specific nasal IgA previously induced in all ponies after the third immunization had blocked attachment of CT-SeMF3 to the nasal mucosa. However, ponies #87, 90 and 94 made no detectable nasal SeM- or CTB-specific IgA or IgG after the first administration yet failed to show an increase in specific serum antibodies after a second administration. It is possible that specific antibodies were induced in nasal secretions of these animals at low, undetectable levels yet in an amount sufficient to neutralize CT-SeMF3 attachment to the nasal mucosa. However, a previous study in mice has suggested that pre-existing high levels of CTB-specific serum and salivary antibodies do not block serum or mucosal responses to subsequent intranasal immunization with a streptococcal antigen conjugated to CTB [16]. It is possible that the differences in responses of horses and mice are explained by the use of a conjugate of CTB and streptococcal peptide in the mice whereas the chimera administered to horses consisted of assembled CTB pentamers and A2-SeMF3 in which the binding activity was possibly more susceptible to antibody neutralization. Another study has shown that oral immunization of mice with CTA2/B genetically linked to serine-rich Entamoeba histolytica surface protein (SREHP) 14 days following the primary immunization boosted SREHP-specific serum IgG responses [17]. However, a third immunization, 14 days after the second, boosted specific intestinal IgA but not serum IgG [17].

Survival of S. equi was significantly reduced in the presence of SeMF3-specific antibodies in sera obtained 2 weeks after the second intranasal administration of CT-SeMF3 when serum titers were at their peak, but improved steadily in sera collected after subsequent administrations. Greater and prolonged reduction in bacterial survival is dependent on high affinity antibodies formed during secondary antibody responses, which were apparently not induced in the present study since specific responses were never boosted. Opsonophagocytic (bactericidal) antibodies are important in clearance of pathogenic streptococci including S. equi from the blood and tissues and are mouse protective [18,19]. Effective vaccines should clearly stimulate high levels of these antibodies and prime memory cells for rapid
anamnestic opsonic responses. It is possible and likely that use of a longer SeM sequence including the N-terminal half of the molecule would have resulted in better bactericidal responses because of the inclusion of a greater number of opsonic B cell as well as T cell epitopes.

Although the primary aim of the project was not to demonstrate protective immunogenicity of the CT-SeMF3 chimera, the results indicate that mucosal and serum antibody specific for the most immunogenic region of SeM, does not protect against streptococci. The absence of protection may be explained by the fact that the protective immunogenic region of SeM is not located on the F3 sequence. Alternatively, one or more other as yet unknown protective immunogens of S. equi are required. Finally, the CT-SeM chimera may not have elicited antibodies with affinity comparable to that in recovered horses resistant to re-infection.

In conclusion, it has been demonstrated that a CT-SeMF3 chimera applied to the equine nasal mucosa is immuno- genic in the absence of intact CT. However, the absence of a booster serum response is a potential limiting factor which requires further study before this mode of vaccine delivery will have practical application in the prevention of streptococci. Overcoming this problem may require that booster intranasal vaccinations be performed in a manner that pro- tects the chimera from the blocking effects of pre-existing antibodies.

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References