Contribution of Each of Four Superantigens to *Streptococcus equi*-Induced Mitogenicity, Gamma Interferon Synthesis, and Immunity

Romain Paillot,* Carl Robinson, Karen Steward, Nicola Wright, Thibaud Jourdan, Nicola Butcher, Zoe Heather, and Andrew S. Waller

Animal Health Trust, Centre for Preventive Medicine, Lanwades Park, Newmarket, Suffolk CB8 7UU, United Kingdom

Received 23 September 2009/Returned for modification 19 October 2009/Accepted 26 January 2010

*Streptococcus equi* is the causative agent of strangles, the most frequently diagnosed infectious disease of horses worldwide. The disease is characterized by abscessation and swelling of the lymph nodes of the head and neck, which can literally strangle the horse to death. *S. equi* produces four recently acquired phage-associated bacterial superantigens (sAgs; SeeH, SeeI, SeeL, and SeeM) that share homology with the mitogenic toxins of *Streptococcus pyogenes*. The aim of this study was to characterize the contribution of each of these *S. equi* sAgs to mitogenic activity *in vitro* and quantify the sAg-neutralizing capacity of sera from naturally infected horses in order to better understand their role in pathogenicity. Each of the sAgs was successfully cloned, and soluble proteins were produced in *Escherichia coli*. SeeL, SeeM, and SeeI induced a dose-dependent proliferative response in equine CD4 T lymphocytes and synthesis of gamma interferon (IFN-γ). SeeH did not stimulate equine peripheral blood mononuclear cells (PBMC) but induced proliferation of asinine PBMC. Allelic replacement mutants of *S. equi* strain 4047 with sequential deletion of the superantigen genes were generated. Deletion of *seeI*, *seeL*, and *seeM* completely abrogated the mitogenic activity and synthesis of IFN-γ, in equine PBMC, of the strain 4047 culture supernatant. Sera from naturally infected convalescent horses had only limited sAg-neutralizing activities. We propose that *S. equi* sAgs play an important role in *S. equi* pathogenicity by stimulating an overzealous and inappropriate Th1 response that may interfere with the development of an effective immune response.

*S. equi* subsp. *equi* is a Lancefield group C streptococcus. It is a host-restricted obligate pathogen of equids and the causative agent of strangles, the most frequently diagnosed infectious disease of horses worldwide (6, 33). Initial infection of the nasopharyngeal mucosal surface is followed within hours by a rapid spread to the draining lymph nodes, where *S. equi* multiplies despite a strong immune response. Infection and inflammation of the submandibular and/or retropharyngeal lymph nodes result in their abscessation and swelling, which can literally strangle the horse to death. In a limited number of cases, *S. equi* spreads systemically, forming abscesses in other organs. This condition, known as “bastard strangles,” is usually fatal to the animal (28).

Analysis of the *S. equi* strain 4047 and *Streptococcus zooepidemicus* strain H70 genomes provided evidence of horizontal genetic exchange between *S. equi*, *S. zooepidemicus*, and *Streptococcus pyogenes* that has affected the pathogenicity of these important bacteria (10). *S. equi* produces four phage-associated bacterial superantigens (sAgs; SeeH, SeeI, SeeL, and SeeM) that share homology with the mitogenic toxins of *S. pyogenes*, a Lancefield group A streptococcus (GAS) that infects humans (5, 25, 26). The prophage δSEQ3 of *S. equi* contains coding sequences (CDSs) for the sAgs SeeM [SPE-M(Sc)] and SeeL [SPE-L(Se)]. SeeL and SeeM are closely related to the sAgs SpeL and SpeM of *S. pyogenes* serotype M18 with MGAS 8232 (29), with DNA sequence homologies of 99% and 98.1% and amino acid sequence identities of 97% and 96%, respectively (1, 26). The prophage δSEQ4 contains genes encoding the previously described sAgs SeeH (SePE-H) and SeeL (SePE-I), which share 98% and 99% amino acid sequence identities with SpeH and SpeL of *S. pyogenes* strain Manfredo, respectively (5). Superantigens from *S. pyogenes* have been extensively studied and are known to affect the virulence of this pathogen. Superantigens are potent immunostimulatory molecules that disrupt innate and adaptive immune responses through nonspecific T-lymphocyte proliferation and the generation of an overzealous proinflammatory response (14, 31). Superantigen activities are based on their abilities to bypass the mechanism of major histocompatibility complex (MHC)-restricted antigen presentation (7). Conventional exogenous antigens are processed and presented by antigen-presenting cells (APC) within the antigen groove of specific MHC class II molecules and are recognized by an antigen-specific T-cell receptor (TCR), which results in a highly specific T-cell activation (1 in 106 T lymphocytes activated). Secreted sAgs bind as intact proteins, directly to the MHC class II molecule outside the peptide-binding site and to one or more specific TCR Vβ chains. Since the number of different Vβ chains is limited in the human T-cell repertoire, a larger fraction (5 to 20%) of the T-cell population can be activated (13, 14). Superantigen-dependent T-cell activation results in the uncontrolled release of proinflammatory mediators and cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-6, and gamma interferon...
were stored at ~70°C in 50% glycerol. The purity of the proteins was assessed by polyacrylamide gel electrophoresis and staining with Coomassie blue. All of the recombinant sAgs migrated at the expected sizes and were shown to be greater than 99% pure.

**Generation of S. equi mutants containing superantigen deletions.** In order to generate single deletion mutants lacking seeH, seeL, or seeM, double deletion mutants lacking seeH and seeL (DH, DL, or DM), a triple deletion mutant lacking seeL and seeM (DLM), and a quadruple deletion mutant lacking seeH, seeL, seeM, and seeM (DLMH) of *S. equi* strain 4047 by allelic replacement, PCR products flanking the sequences to be deleted were generated using Vent DNA polymerase (New England Biolabs) and the relevant primers listed in Table 1. The corresponding PCR products were then digested with the restriction endonucleases EcoRI and HindIII (5′ product) and HindIII and SalI (3′ product) and ligated into the EcoRI- and Sal-digested pG + Host9131 plasmid (19) in a three-way ligation to form the different deletion constructs, pGΔAsAg. Engineering of a HindIII site into primers as part of the cloning strategy results in the introduction of a non-sAg DNA sequence at the site of the deletion. The different pGΔAsAg plasmids were transformed into *E. coli* TG1/pEPα−, and transformants were selected at 37°C on LB plates containing erythromycin (150 μg/ml).

**All-sAg replacement mutagenesis.** Transformation of the encapsulated strain 4047 with the different pGΔAsAg plasmids was achieved using a modification of the method described by Simon and Ferretti (27), as described by Hamilton et al. (8). Allelic replacement of the desired sAg gene(s) was performed as previously described for a *S. pyogenes* mutant (8), and the deletions were confirmed by PCR and DNA sequencing. Absence of sAg expression in *S. equi* mutants was confirmed after DNA extraction by using an RNasey midikit (Qiagen) and analysis by quantitative reverse transcription-PCR (qRT-PCR) with the primers described for the genes encoding the mitogenic superantigens at the indicated concentrations. Equine sAg plasmids were also transformed into primers as part of the cloning strategy results in the introduction of a non-sAg DNA sequence at the site of the deletion. The different pGΔAsAg plasmids were transformed into *E. coli* TG1/pEPα−, and transformants were selected at 37°C on LB plates containing erythromycin (150 μg/ml).

**MATERIALS AND METHODS**

**Streptococcus strains.** *S. equi* strain 4047 was originally isolated in 1990 from a submandibular abscess of a New Forest pony and has been maintained in the culture collection of the Animal Health Trust (Newmarket, United Kingdom). Twenty-eight isolates of *S. equi* (7364, JKS 225, 7325, 7171, 303, 3155, JKS 063, JKS 043, 7329, 3682, CF32, 1351, SA, 8229, 7326, 3156 7235, 4047, 7344, 3154, JK0044, 181063, 1350, 1931, 7060, 7140, and JKS 55, all sequence type 179 [ST-179], and 7329 [ST-151]) were used. Twenty-two of these 28 *S. equi* isolates have been shown to contain seeL, seeL, seeM, and seeM by quantitative PCR (qPCR) (10). Twenty-one isolates of *S. zooepidemicus* (5845 [ST-45], H70 [ST-1], 3512 [ST-143], 8250 [ST-146], 4859 [ST-119], 5770 [ST-106], 4895 [ST-110], 5936 [ST-106], 2410 [ST-144], 8295 [ST-104], 8575 [ST-97], BHS41 [ST-10], 6458 [ST-82], D14a [ST-2], 2958 [ST-178], 5622 [ST-106], 8275 [ST-104], 5768 [ST-112], 8301 [ST-104], 4863 [ST-108], and 4887 [ST-108]) were used in this study. These *S. zooepidemicus* isolates belong to 16 different sequence types (as defined by multilocus sequence typing [MLST] [37]), from which representatives strains were screened for the presence of seeL, seeM, seeM, and seeM (10). Furthermore, all the *S. zooepidemicus* were directly screened for the presence of these four superantigens. seeL and seeM were found to be absent in all *S. zooepidemicus* isolates. The isolates 5936, 5770, 5768, and 5622 contain seeM and seeM, respectively, and were selected to investigate the activity and immunogenicity of SeeM and SeeM.

**Production of recombinant *S. equi* sAgs.** The genes encoding the mitogenic toxins were cloned as glutathione S-transferase (GST) fusions using pGEX-3X and the primers listed in Table 1. The cloned fragments corresponded to codons 26 to 259 (seeH), 33 to 236 (seeL), 27 to 259 (seeM), and 36 to 262 (seeM). In each case the DNA encoding the signal peptide was omitted. PCR products were generated using strain 4047 DNA and Phusion DNA polymerase (New England Biolabs). Purified PCR products were cut with either BamHI and SmaI (seeH and seeH) or BamHI and EcoRI (seeL and seeM), ligated into the pGEX-3X vector cut with the appropriate restriction enzymes, and transformed into Escherichia coli DH10B, and transformants were selected at 37°C on 2X YT (yeast extract-tryptone) plates containing ampicillin (100 μg/ml). For expression, cultures (10 ml) were grown overnight at 37°C in 2X YT with ampicillin (100 μg/ml), diluted 1/10 the next day into 100 ml of 2X YT, grown for 1 h, induced by the addition of 0.5 mM isopropyl-D-thiogalactopyranoside, and grown for a further 4 h at 28°C. Cells were harvested by centrifugation and lysed, and fusion protein was recovered using glutathione-Sepharose beads (Amersham). Factor Xa (Amersham) was then used to cleave the recombinant proteins from the GST tag. The concentrations of purified recombinant sAgs were quantified, and the proteins were used as antigens in the presence of equine peripheral blood mononucleated cells (PBMC) in vitro (2, 5). Both recombinant sAgs were pyrogenic for rabbits after intravenous inoculation, but only SeeH showed pyrogenic activity in ponies (5). Convalescent-phase sera purified from *S. equi*-infected horses possessed antibodies specific to SeeH and SeeM, and convalescent horses or those immunized with SeeH were resistant to SeeH-induced pyrogenic activity in vivo (5). To our knowledge, the activity and immunogenicity of SeeL and SeeM have not been investigated in the horse, and the overall contribution of each of these superantigens to *S. equi* mitogenicity is unknown.

This study investigated the activities of recombinant *S. equi* sAgs and *S. equi* culture supernatants on equine PBMC in vitro. The impact of sequential or full deletion of *S. equi* superantigen genes on T-cell activation with wild-type *S. equi* is also reported for the first time. Finally, the kinetics of *S. equi* sAg antibody responses developed by convalescent horses who have suffered from streptococcal disease and the ability of their sera to neutralize sAg activity in vitro are quantified.
on forward and side scatter (FSC and SSC) characteristics and CD5 staining (17).

The gate used to select PBL during the analysis was based on forward and side scatter (FSC and SSC) characteristics and CD5 staining (17).

Deletion construct primers

<table>
<thead>
<tr>
<th>Expression cloning primers</th>
<th>Encoding residues</th>
<th>Restriction enzymes</th>
<th>Primer sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SceI</td>
<td>26–259</td>
<td>BamHI-SmaI</td>
<td>5’SeeGST-CCCCGGATCTCTAATATCCACGTTACGCG</td>
</tr>
<tr>
<td>SceH</td>
<td>33–236</td>
<td>BamHI-SmaI</td>
<td>5’SeeHGST-CCCCGGATCTCTAATATCCACGTTACGCG</td>
</tr>
<tr>
<td>SceL</td>
<td>27–259</td>
<td>BamHI-EcoRI</td>
<td>5’SeeGST-CCCCGGATCTCTAATATCCACGTTACGCG</td>
</tr>
<tr>
<td>SceM</td>
<td>36–262</td>
<td>BamHI-EcoRI</td>
<td>3’SeeGST-CCCCGGATCTCTAATATCCACGTTACGCG</td>
</tr>
</tbody>
</table>

Deletion construct primers

<table>
<thead>
<tr>
<th>Deletion construct</th>
<th>EcoRI-HindIII-Sall</th>
<th>EcoRI-HindIII-Sall</th>
<th>EcoRI-HindIII-Sall</th>
</tr>
</thead>
<tbody>
<tr>
<td>SceL/SceH</td>
<td>IL1</td>
<td>IL2</td>
<td>IL3</td>
</tr>
<tr>
<td>SceL/SceM</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
<tr>
<td>SceL</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
<tr>
<td>SceH</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
<tr>
<td>SceL</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
<tr>
<td>SceL</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
<tr>
<td>SceM</td>
<td>HI1</td>
<td>HI2</td>
<td>HI3</td>
</tr>
</tbody>
</table>

Primers for quantification of sAg expression

| SceI | Forward, 5’-GGCCGGCTGTCCTGAGATTTTC |
| SceH | Forward, 5’-TGTGGAGTGCCTCAGGAAAAAAC |
| SceL | Reverse, 5’-TGTGGAGTGCCTCAGGAAAAAAC |
| SceM | Forward, 5’-TGTGGAGTGCCTCAGGAAAAAAC |
| GyrA | Forward, 5’-CCATAGAACCAAAAGATTCCTCACATAG |

* Underlined sequences represent engineered restriction sites for BamHI (GGATCC), EcoRI (GAATTC), HindIII (AAGCTT), SalI (GTCGAC), SmaI (CCCGGG), and EcoRI (GAATTC).

The percentage of sAg-specific IFN-γ synthesis was calculated according to the following formula (22): (percent sAg-stimulated IFN-γ synthesis) – (percent medium-stimulated IFN-γ synthesis). The IFN-γ SI was calculated as follows: (experimental IFN-γ synthesis)/(control IFN-γ synthesis).

Measurement of sAg-specific antibody response by ELISA and neutralizing activity.

Microtiter plates (96-well Immulon 2HB; Dynex Technologies) were coated overnight at 4°C with 100 µl of recombinant S. equi sAgs at a concentration of 2 µg per ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6; Sigma-Aldrich). Plates were washed four times with 400 µl of PBS plus 0.05% (vol/vol) Tween 20 (PBS-T). The plates were blocked with 300 µl of PBS-T plus 5% (vol/vol) Marvels skimmed milk per well for 1 h at 37°C and then washed four times with 400 µl of PBS-T. Serum samples (100 µl) diluted 1/800 in PBS were
added to the wells. Plates were incubated at 37°C for 1 h and washed again four times with 400 μl of PBS-T. Detection of superantigen-specific bound antibodies was performed with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (Bethyl Laboratories) diluted 1/600 in PBS-T plus 1% (vol/vol) Marvels skimmed milk. Plates were incubated for 1 h at 37°C and then washed six times with 400 μl of PBS-T. HRP was detected by incubation with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate (Kirkegaard & Perry Laboratories) at room temperature for 10 min. The reaction was stopped by the addition of 100 μl of H2SO4 (0.18 M). Results are expressed as the optical density (OD) at 450 nm. For the SeeL- and SeeM-specific ELISAs, the cutoff values (0.25 and 0.68 OD, respectively) were defined as the 2-fold average day zero absorbance (sample prior to S. equi infection). To measure neutralizing activity of equine sera, 5 μl of serum was added (final volume, 200 μl). The percentage of neutralization was calculated according to the following formula: 100 − [(proliferation in the presence of convalescent-phase serum × 100)/(proliferation in the presence of preinfection serum)].

**Statistical analyses.** Statistical analyses were performed with the StatGraphics Plus program for Windows. Analysis of variance (ANOVA) was used to test the significance of data between groups (Tukey’s honest significant difference test or Bonferroni procedure with a confidence interval of 95%). The level of significance was set at a P value of <0.05. If variances between each group were not homogenous (P < 0.05), data were log transformed to allow ANOVA to be applied.

**RESULTS**

*S. equi* sAg-induced lymphoproliferation *in vitro.* CFSE-stained equine PBMC were cultivated in the presence of recombinant *S. equi* sAgs or medium alone as negative controls for 3 days before being analyzed by flow cytometry (Fig. 1A). SeeI, SeeL, and SeeM repeatedly induced proliferation of...
TABLE 2. Proliferative responses of equine and asinine PBMC to S. equi sAgS at 0.125 μg/ml

<table>
<thead>
<tr>
<th>Animal</th>
<th>SI with indicated sAg</th>
<th>SeeI</th>
<th>SeeH</th>
<th>SeeL</th>
<th>SeeM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pony 1</td>
<td>10.0 ± 0.9</td>
<td>1.2 ± 0.3</td>
<td>33.0 ± 1.7</td>
<td>28.2 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>Pony 2</td>
<td>11.4 ± 1.1</td>
<td>1.0 ± 0.3</td>
<td>16.0 ± 0.6</td>
<td>19.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Horse 1</td>
<td>12.4 ± 1.5</td>
<td>1.3 ± 0.2</td>
<td>22.3 ± 3.5</td>
<td>31.5 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Horse 2</td>
<td>3.8 ± 1.1</td>
<td>0.5 ± 0.2</td>
<td>7.4 ± 1</td>
<td>12.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Horse 3</td>
<td>15.8 ± 3.3</td>
<td>0.7 ± 0.1</td>
<td>32.3 ± 2.7</td>
<td>63.4 ± 7</td>
<td></td>
</tr>
<tr>
<td>Horse 4</td>
<td>5.7 ± 0.7</td>
<td>0.9 ± 0.4</td>
<td>17.7 ± 3.3</td>
<td>9.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Horse 5</td>
<td>6.1 ± 0.0</td>
<td>1.6 ± 0.5</td>
<td>4.7 ± 0.1</td>
<td>6.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Horse 6</td>
<td>5.6 ± 0.7</td>
<td>1.0 ± 0.1</td>
<td>11.7 ± 0.4</td>
<td>17.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Donkey 1</td>
<td>14.8 ± 1.2</td>
<td>6.1 ± 0.2</td>
<td>44.3 ± 13.3</td>
<td>66.1 ± 13.6</td>
<td></td>
</tr>
<tr>
<td>Donkey 2</td>
<td>4.9 ± 0.4</td>
<td>3.1 ± 0.8</td>
<td>32.9 ± 0.1</td>
<td>38.4 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>Donkey 3</td>
<td>2.5 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>7.1 ± 1.2</td>
<td>8.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Donkey 4</td>
<td>2.8 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>8.1 ± 0.4</td>
<td>8.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Donkey 5</td>
<td>9.1 ± 0.3</td>
<td>3.1 ± 0.3</td>
<td>19.0 ± 3.3</td>
<td>20.5 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Donkey 6</td>
<td>9.4 ± 0.4</td>
<td>5.0 ± 0.2</td>
<td>29.4 ± 2.0</td>
<td>31.1 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Donkey 7</td>
<td>7.9 ± 0.3</td>
<td>1.9 ± 1.0</td>
<td>14.9 ± 0.5</td>
<td>13.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Donkey 8</td>
<td>19.9 ± 0.7</td>
<td>16.6 ± 3.1</td>
<td>29.2 ± 1.5</td>
<td>46 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

* Equine PBMC (2 × 10^5) were cultured for 4 days in triplicate with sAgS and incubated with [3H]T for 16 h before quantification of proliferation. Results are presented as the SI, and positive responses (SI > 2) are shown in bold. PBMC purified from horses or ponies were used as controls.

Culture supernatants from 28 S. equi isolates were screened for stimulation of IFN-γ synthesis and mitogenic activity when cultured in the presence of equine PBMC. Culture supernatants from S. zooepidemicus isolates (n = 18) that were negative for mitogenic activity were used as controls. As illustrated in Fig. 3, all S. equi isolates stimulated IFN-γ synthesis after 24 h of incubation. The average IFN-γ SI for S. equi supernatants was 8.5 ± 2.3 (n = 28) and was 1.3 ± 0.7 for S. zooepidemicus supernatants (n = 18). Repartition of the IFN-γ SI induced by S. zooepidemicus culture supernatants (n = 18) revealed a cutoff value of 3.1 (upper limit of distribution containing 99% of results) above which results were considered positive. Four culture supernatants from S. zooepidemicus isolates, which were positive for mitogenic activity, were also found to be positive for IFN-γ synthesis (Fig. 3B).

Deletion of sAg genes abrogates mitogenic activity and IFN-γ synthesis induced by S. equi culture supernatants. Equine PBMC proliferation and IFN-γ synthesis (Fig. 4A and B, respectively) were measured after stimulation with culture supernatants from wild-type strain 4047 (control) or S. equi mutants with superantigen deletions. As illustrated in Fig. 4, the impact of single sAg deletion (ΔI, ΔH, ΔL, or ΔM) on S. equi supernatant-induced cellular responses was limited, with only the seeL deletion resulting in a statistically significant inhibition of lymphoproliferation and IFN-γ synthesis (19.1 ± 12.9 and 53.3 ± 16.4, respectively). The combined deletion of seeI and seeM (ΔIΔM) significantly increased the inhibition of both lymphoproliferation and IFN-γ synthesis (62.8 ± 17.8 and 84 ± 12, respectively), compared to single deletion alone or the seeH and double deletion strain (ΔHI). Deletion of seeL, seeI, and seeM (ΔIΔLM) or all sAg genes (ΔIΔHΔLΔM) entirely abrogated mitogenic activity and IFN-γ synthesis induced by S. equi culture supernatants compared with wild-type strain 4047.

Infection with S. equi induced a sAg-specific antibody response in vivo. Convalescent-phase sera have been shown to contain antibodies to both SeeL and SeeH. Furthermore, horses that recover from strangles or are immunized with SeeI are resistant to the pyrogenic effects of See1 (2, 5). The antibody response to all four S. equi sAgS was measured by ELISA in seven horses during strangles outbreaks (Fig. 5). The initial sera (day zero) were taken from healthy horses and were negative for the presence of S. equi sAg-specific antibodies (with the exception of horse number 7, which presented low levels of SeeH- and SeeL-specific antibodies). It is assumed that these horses had either not yet been naturally infected with S. equi or were at an early stage of natural infection (within a few days) at day zero. While the precise date of infection is not known, these horses were diagnosed with S. equi infection a few days after the initial serum sample had been taken. SeeL- and SeeH-specific antibody levels rose quickly above the background cutoff thereafter. The peak of the antibody response for each horse was between 20 and 80 days after the first sample. The antibody responses to SeeL and SeeM were less homogenous, with three and two horses that did not seroconvert to SeeM and SeeL, respectively.

Convalescent-phase sera have limited neutralizing sAg activity in vitro. Equine PBMC were cultured with recombinant
SeeI, SeeL, and SeeM (concentration close to 10 times the calculated $P_{50}$) that were incubated with sera (5 μl) purified from six horses prior to and after S. equi infection (convalescent-phase sera). Convalescent-phase sera consistently reduced SeeI-induced proliferation (0.5 ng/ml) with a positive correlation ($R^2 = 0.84; n = 11$) between the SeeI antibody titer and the percentage of neutralization (Fig. 6A). The abilities of convalescent-phase sera to neutralize recombinant SeeL- and SeeM-induced lymphoproliferation (1 ng/ml and 10 pg/ml, respectively) varied between individuals, as illustrated in Fig. 6B.
and C. SeeL- and SeeM-specific antibodies were measured in horses 2 to 5 after S. equi infection. Only sera from horses 2 and 5 showed some levels of neutralizing activity. No correlations were found between SeeL or SeeM antibody titers and the percentage of neutralization ($R^2 = 0.24$ and 0.55, respectively). Convalescent-phase sera from horses 3 to 5 were further tested against culture supernatants from wild-type strain 4047 and ΔHI and ΔLM deletion mutants. Inhibition of SeeI-induced lymphoproliferation by convalescent-phase sera was confirmed when culture supernatant from ΔLM S. equi mutants was used (diluted 1/20) to stimulate equine PBMC (data not shown). Convalescent-phase serum from horse number 5 was the only sample to significantly inhibit proliferation induced by all culture supernatants tested (data not shown).

**DISCUSSION**

The cell culture supernatant of S. equi has been shown previously to induce proliferation of equine PBMC in vitro (2). This mitogenic activity is due to the production of four sAgs by S. equi. A genome analysis of S. equi strain 4047, S. zooepidemicus H70, and S. pyogenes strains revealed that S. equi gained these sAgs through the acquisition of two prophages (φSEQ3 and φSEQ4) (10). SeeH (SPE-H) and SeeI (SPE-I) have been previously characterized and studied in the horse (5). SeeL [SPE-L(Se)] and SeeM [SPE-M(Se)] have been identified, but their in vitro mitogenic activities on equine cells were not investigated (1, 26). The aim of this study was to better understand the contribution made by each of the four S. equi sAgs toward S. equi pathogenicity.

Each of the S. equi sAgs was cloned successfully, and soluble proteins were produced in E. coli. Three of the recombinant sAgs (SeeI, SeeL, and SeeM) induced a strong dose-dependent proliferative response and synthesis of IFN-γ in equine T lymphocytes. The kinetics of lymphoproliferation was similar to the kinetics described by Anzai et al. (2), with proliferation observed between the second and fifth days of culture. The kinetics of proliferation induced by SeeI was delayed in time. This is explained by a lower frequency of cells stimulated by SeeI. S. equi sAgs mainly stimulated CD5$^+$ CD4$^-$ and, to a lesser extent, CD5$^+$ CD8$^-$ lymphocytes. Stimulation of CD8$^+$ T lymphocytes by streptococcal sAgs has been previously reported (20, 35). SeeH was inactive when incubated with equine PBMC purified from several different breeds of horses (Welsh Mountain ponies, thoroughbreds, Gypsy cobs, and Shetland ponies). However, SeeH induced the in vitro proliferation of PBMC from six out of eight donkeys. This result confirmed that the absence of SeeH activity when cultured with equine PBMC was not due to the production of a nonfunctional protein but could be explained by the absence of equine cells harboring the appropriate TCR V\beta chains and/or MHC class II molecules recognized by SeeH. This result contradicts a previous report that demonstrated in vitro mitogenic activity of recombinant SeeH on equine PBMC purified from Welsh ponies (5). However, our results are consistent with these authors’ observation that SeeH did not induce pyrexia in three ponies. The absence of mitogenic activity in culture supernatant from the ΔLM1 mutant S. equi, which expresses seeH RNA but not seeL, seeM, or seeR (data not shown), supports the absence of proliferation induced by recombinant SeeH. The SeeH mitogenicity observed using donkey PBMC illustrates that the acquisition of a broad range of sAgs may increase the ability of S. equi to infect several equid species and argues that these hosts may differ in their susceptibility to sAgs through sequence variation of MHC class II and TCRs. HLA class II polymorphisms are known to influence the nature of T-cell responses to S. pyogenes sAgs (15) and the risk of severe streptococcal infection in humans (11). One may speculate that recent acquisition of SeeH and SeeI by S. equi, possibly from S. pyogenes, and functional redundancy through the production and activity of SeeI, SeeL, and SeeM, has not led to the opportunity or selective pressure for the adaption of SeeH to equine MHC and TCR. The equine genome has been recently sequenced (36), opening up the opportunity to identify the equine V\beta chains recognized by S. equi sAgs and to quantify
the risks associated with the production of particular alleles thereof. The donkey genome has not yet been sequenced. Our data suggest that analysis of the MHC class II receptors encoded will help to explain the differences in SeeH activity in the treatment of equine and asinine PBMC.

This report is the first demonstration of IFN-γ synthesis by $T CD5^-$CD4$^+$ T lymphocytes following treatment with S. equi sAgs. Superantigen-induced IFN-γ synthesis was time dependent and detectable after a few hours of incubation with SeeI, SeeL, and SeeM. The cytokine response peaked during the first two days of culture and decreased quickly thereafter. Similar IFN-γ synthesis kinetics have been described in human PBMC stimulated in vitro with S. pyogenes SmeZ (35). Interestingly, IFN-γ synthesis and proliferation induced by S. equi sAgs were different in terms of dose response and kinetics, with a rapid cytokine response induced by relatively high concentrations of sAgs compared with T-cell proliferation, which was delayed but required very low concentrations of recombinant.sAgs.

FIG. 4. Box-and-whisker plot histograms of cell proliferation (A) and IFN-γ synthesis (B) induced by S. equi culture supernatants after sequential deletion of sAgs. (A) For cell proliferation, $2 \times 10^5$ equine PBMC were cultured for 4 days in triplicate with culture supernatants (1/20) and incubated with ³HT for 16 h before measurements of proliferation. Results are expressed as the percentage of proliferation inhibition compared with the mitogenic activity of wild-type S. equi strain 4047. (B) For IFN-γ synthesis, $1 \times 10^6$ equine PBMC were incubated overnight with culture supernatants (1/20). IFN-γ synthesis was detected by flow cytometry after intracellular staining. Results are expressed as the percent inhibition of IFN-γ synthesis compared with wild-type strain 4047. Differences between groups are indicated with a letter code (a to d). For example, a and b indicate a statistical difference ($P < 0.05$) between the two groups, and a group noted with ab is not statistically different from a group noted a or b ($P > 0.05$). The most relevant $P$ values are indicated. The rectangle represents 50% of the observed response for each group, the horizontal line indicates the median of the group, and the cross indicates the average of the group. Squares indicate outlier results. n indicates the number of samples measured per group.
sAgs. Sriskandan et al. demonstrated that IFN-γ synthesis and T-cell proliferation were independent of each other (30). IL-12 has been often identified as the cytokine regulating IFN-γ synthesis after stimulation with bacterial sAgs (30, 32). In the current study, the addition of BFA, which inhibits the Golgi apparatus and prevents protein secretion or expression on the cell surface and therefore IL-12 secretion, at the start of the incubation with cell culture supernatants or recombinant sAgs indicated that the synthesis of IFN-γ was the consequence of direct T-cell activation by sAgs, presumably subsequent to binding to MHC class II and TCR molecules. Further study is necessary to investigate the regulation of IFN-γ synthesis after stimulation with S. equi sAgs.

The combined use of the proliferation assay and detection of intracellular IFN-γ synthesis has proved to be useful for the screening of S. equi and S. zooepidemicus strains for the presence or absence of sAg production. A proliferation SI between 1.5 and 2.5 was often difficult to interpret due to the heterogeneity of the PBMC proliferative response between individuals. In the current study, several isolates of S. equi induced a proliferation SI between 1.5 and 2.5 but were clearly positive for the synthesis of IFN-γ. Such results highlight these specific isolates and strains for further investigation in order to confirm the presence of sAgs and to determine their level of expression. Differences in sAg production may occur between S. equi strains and could provide an explanation as to why some of these strains are associated with increased severity of disease. S. zooepidemicus strains have a very diverse profile of mitogenic activity (10). Detection of IFN-γ synthesis may assist the identification of sAg-producing strains and the ability to dis-

![Figure 5](http://iai.asm.org/) Kinetics of S. equi sAg-specific antibody responses in vivo. The antibody responses to all four S. equi sAgs were quantified by ELISA using sera taken from seven horses during strangles outbreaks. The initial sera (day zero) were taken from healthy horses with no signs of disease. These horses were diagnosed with strangles infection a few days after the initial serum samples were taken. Dotted lines represent the threshold above which the antibody response was considered positive.
criminate between positive and negative strains. In the current study, four *S. zooepidemicus* isolates showed mitogenic activity and stimulated IFN-γ synthesis. They belong to the multilocus sequence type 106 (10) and contain homologues of *seeL* and *seeM*, based on qPCR.

Several elements support the hypothesis that sAgs may disrupt or misdirect the development of productive immune responses to *S. equi*, conferring an advantage by delaying pathogen clearance and increasing the possibility of abscessation and transmission. IFN-γ was produced by equine T lymphocytes.
shortly after in vitro stimulation with *S. equi* sAgs. The synthesis of IFN-γ (a Th1 cytokine) is a key element of the equine type 1 immune response to several intracellular pathogens, including *Rhodococcus equi*, equine herpes virus type 1, and equine influenza virus (9, 16, 22–24). IFN-γ activities against extracellular pathogens such as *S. equi* are unknown in the horse and may be detrimental to the development of protective immunity. Arad et al. (3, 4) demonstrated that the use of superantigen antagonist peptides blocked Th1 cytokine synthesis, prevented staphylococcal enterotoxin B (SEB)-induced lethal toxicity in mice, and allowed the development of a neutralizing antibody-based cross-protective immunity to subsequent challenge with SEB, streptococcal pyrogenic exotoxin A (SPEA), and toxic shock syndrome toxin 1 (TSST-1). In the horse, sAgs are natural targets of the immune response. Artiushin et al. measured SeeH- and See-specific antibodies in poststrangles convalescent-phase sera (5). Poststrangles convalescent-phase equine sera reduced the proliferation of equine PBMC purified from two ponies following treatment with *S. equi* culture supernatants by 80 to 90% (2). The development of an antibody response specific to SeeL and SeeH was confirmed in the current study. The generation of antibody responses to SeeL and SeeM was demonstrated for the first time here, although the strength of antibody response varied between individuals. SeeM and SeeL are genetically linked and time here, although the strength of antibody response varied
time between individuals. SeeM and SeeL are genetically linked and time here, although the strength of antibody response varied
time here, although the strength of antibody response varied
time here, although the strength of antibody response varied
time here, although the strength of antibody response varied
time here, although the strength of antibody response varied
between individuals. SeeM and SeeL are genetically linked and share the same promoter. Therefore, the absence of detectable antibodies against SeeL and/or SeeM in several horses (e.g., horse 6) could be explained by an absence of their expression by the infecting strain in vivo. The regulation of *S. equi* sAg expression requires further investigation. The neutralizing activity of this antibody response was investigated and found to be effective consistently against SeeL and the ΔLM culture supernatants in vitro. However, poststrangles convalescent-phase sera had limited effects on T-cell activation induced by SeeL or SeeM. The overall neutralizing activity of the six convalescent-phase sera purified from thoroughbreds naturally infected with *S. equi* during a United Kingdom outbreak of strangles was at best limited when tested in this study against wild-type strain 4047 culture supernatant (data not shown). This result supports sAg interference of the development of a neutralizing antibody response. However, disruption of the immune response is not necessarily the best survival strategy for *S. equi*, and most horses recovering from strangles usually develop long-term protective immunity (34), which may be based on a strong mucosal response able to counteract *S. equi* at an early stage of the infection. Abscessation of retropharyngeal lymph nodes followed by their rupture into the adjacent guttural pouches (specific features of the horse anatomy consisting of two empty cavities situated at the back of the horse’s head and connected to the upper respiratory tract through the eustachian tubes) has been linked to the development of persistent *S. equi* infection and recurrence of strangles outbreaks. The production of sAg is likely to play a significant role in this process and assist the transmission of *S. equi*. Further investigations will be necessary to quantify the importance of sAgs to the virulence of *S. equi* and to the establishment of carriers.

Results obtained with recombinant *S. equi* sAgs were confirmed in vitro with the use of culture supernatants from the *S. equi* 4047 strain after full or partial deletion of superantigen genes. To our knowledge, this is the first time that the use of multiple sAg gene deletions in streptococci has been reported. Both lymphoproliferation and IFN-γ synthesis were abrogated when *seeL*, *seeM*, and *seeH* were deleted. Furthermore, deletion of *seeL* and *seeM* had a greater impact on superantigen-induced activation than the deletion of *seeL* and *seeH*, confirming that SeeL and SeeM have a higher potency and target a larger percentage of equine PBMC.

In summary, *S. equi* produces four sAgs. Three of these (*SeeL, SeeL*, and *SeeM*) stimulated equine PBMC proliferation and IFN-γ synthesis in vitro, while *SeeH* was active on donkey PBMC only. These results were confirmed using partial or multiple sAg deletion mutants of *S. equi*. Naturally infected horses developed an antibody response that contained limited sAg-neutralizing activity. The analysis of *S. equi* sAgs in the natural host may assist in the understanding of the role of sAgs during streptococcal infection of other hosts, including humans.

ACKNOWLEDGMENT

This work was supported by the Animal Health Trust (United Kingdom).

REFERENCES


Editor: R. P. Morrison

Vol. 78, 2010

IMMUNE RESPONSE TO S. EQUI SUPERANTIGENS 1739


