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RESEARCH ARTICLE

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The identification of MacSe in *Streptococcus equi* ssp. *equi*

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Abstract *Streptococcus equi* subsp. *equi* (*S. equi* ssp. *equi*) causes equine strangles, a highly contagious and widespread purulent lymphadenitis of the head and neck. We have identified MacSe, a novel protein of *S. equi*, by screening a phage library of 3–8 kb random DNA fragments of *S. equi* CF32. MacSe shares 62% and 67.5% amino acid homology with Mac5005 and Mac8345 of *S. pyogenes* respectively. Expression during infection was shown by strong reactivity of the protein with convalescent sera and mucosal wash IgA of ponies infected by commingling exposure. Release into the culture medium was detected during the log phase of growth. Dose dependent anti-phagocytic activity for equine neutrophils involved interaction of MacSe with C3 and neutrophils.

Keywords *Streptococcus equi* ssp. *equi*, MacSe, anti-phagocytic activity

1 Introduction

Streptococcus equi ssp. *equi* of Lancefield group C causes equine strangles, a highly contagious purulent lymphadenitis of the head and neck. Following ingestion or inhalation by a susceptible horse, the organism enters the nasopharyngeal and oropharyngeal tonsils and replicates extracellularly over the following 4–6 d forming long chains arranged singly or in microcolonies. Large numbers of neutrophils infiltrate infected tissues and efferent mandibular or retropharyngeal lymph nodes to which infection has spread; however, they fail to ingest the invading and replicating bacteria (Sheoran et al., 1997; Timoney, 2004). Survival of *S. equi* in these locations depends on subversion of complement mediated opsonization and phagocytosis–both of which are critical features of the innate immune system. Several streptococcal molecules participate in the anti-phagocytic process, the most important of which is SeM, an acid resistant molecule that projects from the cell surface as fibrillae composed of two molecules coiled around each other. The antiphagocytic action of SeM is due to the binding of fibrinogen to its N-terminal half and IgG to its central region, thus hindering access of receptors on neutrophils to C3b on the bacterial surface (Boschwitz and Timoney, 1994a, 1994b). The hydrophilic hyaluronic acid capsule is another major virulence determinant that may function by enhancing the surface presentation and functionality of the hydrophobic SeM and by blocking access of phagocytic receptors to opsonic C3 components on the bacterial surface (Cunningham, 2000). The Lancefield group A S. pyogen produces additional antiphagocytic factors including the Mac5005 protein that directly or indirectly affects phagocytosis. Mac5005 is a homologue of the alpha subunit of CD11b (Mac l), which is a neutrophil and macrophage receptor for C3bi, inhibiting opsonophagocytosis and killing *S. pyogenes* by neutrophils (Lei et al., 2001, 2002). These studies also reveal that *S. equi* ssp. *equi* 4047 (www.sanger.ac.uk) has a Mac homologue whose amino acid sequence is 62.4% identical with that of Mac5005. No other information is provided.

In our study, the Mac protein of *S. equi* ssp. *equi* CF32 was expressed and characterized by screening an expression library of genomic DNA with convalescent horse serum. It also showed that MacSe was secreted during the log phase of growth, eliciting serum and mucosal antibodies in infected horses, binding equine C3, and producing dose dependent inhibition of PMN phagocytic activity for *S. equi* yet, unlike the Mac5005 of *S. pyogenes*, was also bound directly to PMNs.

2 Materials and methods

2.1 Bacterial strains, media and serum

Streptococcus equi ssp. *equi* CF32 and *S. equi* ssp. *zooepidemicus* W60 (Timoney et al., 1985, 1995) were

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cultured in Todd Hewitt Broth (THB) + 0.2% yeast extract at 37°C overnight. *Escherichia coli* strains XL-l Blue MRF' and SOLR (Stratagene, La Jolla, CA) were hosts for phage manipulation and plasmid excision. NovaBlue and BL21 (DE3), (Novagen, Madison, Wis) were used for cloning and expression of recombinant proteins and cultured according to the manufacturer's protocol. Rabbit antiserum to Mac5005 of *S. pyogenes* was kindly provided by Lei et al. (2001, 2002).

2.2 Convalescent sera and nasal washes

Sera and nasal washes were collected at intervals from 6 ponies following infection with *S. equi* ssp. *equi* as described previously (Sheoran et al., 1997, 2002).

2.3 Library screening and plasmid rescue

Library screening and plasmid rescuing methods as reported previously (Artiushin et al., 2004; Verma et al., 2005), were followed with modifications as necessary. A lambda ZAPII library containing 3–8 kb fragments of genomic DNA of *S. equi* ssp. *equi* randomly digested by Tsp5091 was screened with pooled convalescent pony sera pre-absorbed with *E. coli* strains XL-l Blue MRF'. Plaques containing reactive proteins were identified using mouse Mab (CVS39) to equine IgGb followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG phosphatase (Sigma) with BCIP/NBT (Sigma) as substrate. Reactive plaques were picked, replated, and rescreened until all gave a positive signal with the equine antiserum. The phages were analyzed by immunoblotting to confirm expression and to estimate protein size. Following propagation on lawns, *E. coli* XL-1 MRF' plaques were transferred in duplicate to IPTG (Isopropyl-β-D-thiogalactopyranoside) -saturated nitrocellulose disks and immunoblotted with the convalescent serum pool diluted to 1∶500.

2.4 Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in an X-Cell SureLock Mini-Cell (Invitrogen, Carlsbad, Calif.) for 2 h at 125 V in Tris-glycine running buffer (25 $mmol \cdot L^{-1}$ Tris, 192 $mmol \cdot L^{-1}$ glycine, 0.1% SDS (pH 8.31)). Samples for electrophoresis were mixed with l/5 volume of 5×gel loading buffer (100 $mmol \cdot L^{-1}$ Tris-Cl (pH 6.8), 10% SDS, 50% glycerol, 500 $mmol \cdot L^{-1}$ dithiothreitol, and 0.1% bromophenol blue) and boiled for 5 min before loading. The gels were rinsed twice in distilled water and stained with Protoblue Safe Stain (Atlanta, Georgia). Proteins were also transferred to nitrocellulose (0.2 $mmol \cdot L^{-1}$ pore size; Schleicher & Schuell, Keene, N-H.) and blocked with 4% dry milk in Tris-buffered saline (20 $mmol \cdot L^{-1}$ Tris, 150 $mmol \cdot L^{-1}$ NaCl, 0.05% Tween 20 (pH 7.5)). The membranes were incubated with antiserum, followed by Protein G conjugated to horseradish peroxidase (Zymed, San Francisco, Calif.). Bound conjugate was detected using 4-chloro-l-naphthol (Sigma, St. Louis, MO.).

2.5 DNA sequencing and analysis

Plasmids were excised from selected recombinant phages using the ExAssist helper phage and *E. coli* SOLR were isolated by using the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.) and sequenced using standard T7 and Ml3 reverse and custom design primers. Sequencing was performed in a commercial DNA sequencing facility (DavisSequencing LLC, Davis, Calif.), and editing was done with Chromas 1.61. Nucleotide sequences were aligned and connected using DNASIS (Hitachi Software Engineering America, Ltd., San Diego, Calif.). Analyses of nucleotide sequence and deduced amino acid sequences were performed with DNASIS and the Genetics Computer Group package of programs (WisconsinPackage version 10.0; Genetics Computer Group, Madison, Wis.). Homologies were identified by a BLAST search with the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6 Protein expression

Primers MF (GCGCTCGAGGACGATTACCAAAGGAATGC) and MR (GCGGATCCTAATGATGACTGGTTAGC), including an *Xho*I restriction enzyme site and a *Bam*HI restriction site respectively, were designed using DNASIS. The sequence encoding MacSe was amplified by PCR from a rescued clone, which was denatured at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 59°C for 45 s, and 72°C for 1 min and 10 s. The product and PET-15b (Novagen) were digested with *Xho*I and *Bam*HI and then ligated together. The resulting construct was transformed into *E. coli* BL21. Expression of MacSe was induced with 1 $mmol \cdot L^{-1}$ IPTG when the culture reached an optical density of 0.6 at 600 nm, and cells were harvested after 3 h. Recombinant His6-MacSe was isolated using TALON metal affinity resin (Clontech Laboratories, Inc., Palo Alto, Calif.) under denaturation conditions. Fractions containing His6-MacSe were combined and dialyzed against 20 $mmol \cdot L^{-1}$ Tris – 50 $mmol \cdot L^{-1}$ NaCl buffer (pH 7.5). Recombinant MacSE was stored at −70°C prior to use.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Polystyrerte plates (Becton Dickinson, NY) were coated with 5 μg protein overnight followed by blocking with 4% nonfat dry milk. Equine sera (1∶1000) and nasal washes (1∶100) were added to separate wells in triplicate and incubated for 1.5 h at 37°C. Bound immunoglobulin (IgG) was detected with HRP-protein G (Zymed, San Francisco,

CA) and IgA with equine α-chain-specific monoclonal antibody (Mouse BVS-2) followed by HRP conjugated goat anti-mouse serum. Plates were developed with phenylenediamine (Sigma, St. Louis, MO). Secretion of MacSe was measured by coating wells with 20 μL of culture supernatant of *S. equi* CF32 followed by MacSe specific rabbit antiserum (1 : 500) and HRP-goat anti-rabbit IgG. Culture supernatants and mutanolysin extracts of other isolates of *S. equi* ssp. *equi* and *Streptococcus equi* ssp. *zooepidemicus* were similarly screened.

2.8 Polyclonal antiserum

New Zealand white rabbits were injected subcutaneously with 100 μg of recombinant protein and 1 μg of N-acetylmutamyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, MO) adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY). Booster injections with 50 μg protein were performed on day 14 and 28. Serum was collected 35 d after the primary immunization.

2.9 Endopeptidase activity of MacSe

The ability of MacSe to cleave immuoglubin was studied by incubating 30 μg equine IgGb, IgA and IgM with 1 μg MacSe in phosphate-buffer saline at 37°C for 2 h and analyzing the reaction mixture by SDS-PAGE (Lei et al., 2001).

2.10 Biotinylation of recombinant MacSe

Recombinant MacSe was biotinylated with Sulfo-NHS-LC biotin following the manufacturer's protocol. Excess biotin was removed by gel filtration through a PD-10 column (Amersham-Pharmacia) (Pierce, IL). The concentration of biotinylated MacSe protein was measured with BCA Kit.

2.11 Binding of MacSe to PMNs

Fresh PMNs (2×10^6) isolated by Percoll gradient were incubated with 30 μg biotinylated MacSe at 37°C for 1 h. The PMNs were then washed three times with PBS and sonicated. Binding of biotinylated MacSe to PMNs was assayed by ELISA with HRP-avidin. Neutrophils were incubated with the biotinylated MacSe protein in phosphate buffer solution (PBS) for 1 h at 4°C or 37°C, and washed 3 times in PBS. They were then incubated with FITC-conjugated streptavidin (Pierce) for 30 min at 4°C, washed 3 times and fixed in PBS-1% formaldehyde. Cells were analyzed on a FACS calibur flow cytometer equipped with Cell Quest Pro software. Data from 15000 events were acquired for each staining. The positivity threshold was defined such that $\leqslant 1\%$ control cells were positive.

2.12 Binding of MacSe to C3

Log-phase cultures of *S. equi* ssp. *equi* CF32, E23 and Irish 1 in THB + 0.2% yeast extract were washed three times and incubated with fresh serum from a horse never exposed to *S. equi* ssp. *equi* for 15 min at 37°C. After three washes, biotinylated MacSe was added and incubated at 37°C for 1 h. One mol·L^{-1} hydroxylamine was added to the bacterial pellets after complete washing, biotinylated MacSe in the sonicated was assayed by ELISA as the above described.

2.13 Phagocytosis assay

Phagocytosis of *S. equi* ssp. *equi* CF32 by PMNs was performed using a modification of a previously described procedure (Boschwitz and Timoney, 1994; Lei et al., 2002). CF32 was grown to the late exponential phase and opsonized with SeM specific rabbit antiserum for 1 h at 37°C, PMNs (2×10^6) and opsonized CF32 (1×10^7 CFU) were combined in the presence of MacSe, at 0.5 μg·mL^{-1}, 5 μg·mL^{-1}, and 20 μg·mL^{-1}. A control without MacSe was also included. Mixtures were rotated for 1 h at 37°C and phagocytosis was terminated by placing the suspension on ice. Aliquots of each suspension taken at 0 and 60 min were plated on CNA blood agar and colonies were counted after overnight incubation to determine the number of viable bacteria. Bactericidal activity was calculated as a percentage of the count of viable *S. equi* ssp. *equi* at 0 and 60 min.

3 Results

3.1 Isolation and characterization of MacSe

Fifty-one clones strongly reactive with the pool of convalescent serum were isolated. Three expressed a protein of about 40 kDa. Sequence analysis showed that each of these colonies contained the same 1101-bp open reading frame encoding a 367 aa protein with a deduced molecular mass of 41.077 kDa, and a 51-aa signal sequence. The molecular weight of the mature protein was calculated as 35.559 kDa. The amino acid sequence of MacSe shared 62.4% and 67.5% identity with Mac5005 and Mac8345 of *S. pyogenes* respectively. The deduced amino acid sequence of MacSe is shown in Fig. 1.

3.2 Expression and purification of recombinant MacSe

Recombinant MacSe before and after purification by nickel exchange chromatography is shown in Fig. 2. The reactivity of recombinant protein with the convalescent serum pool is also shown.

MSLTLFNDNITITRKGYMKTIAYPNKPHSLSAGLLTAIAIFSLASSNITYADDYQRNATEAYAKEVPHQITSVWTK
GVTPLTPEQFRYNNEDVIHAPYLAHQGWYDITKAFDGKDNLLCGAATAGNMLHWWFDQNKTEIEAYLSKHPE
KQKIIFNNQELFDLKAAIDTKDSQTNSQLFNYFRDKAFPNLSARQLGVMPDLVLDMFINGYYLNVFKTQSTDV
NRPYQDKDKRGGIFDAVFTRGDQTTLLTARHDLKNKGLNDISTIIKQELTEGRALALSHTYANVSISHVINLWGA
DFNAEGNLEAIYVTDSDANASIGMKKYFVGINAHRHVAISAKKIEGENIGAQVLGLFTLSSGKDIWQKLS.

Fig. 1 The deduced amino acid sequences of MacSe
Note: Boxed sequence means a signal sequence.

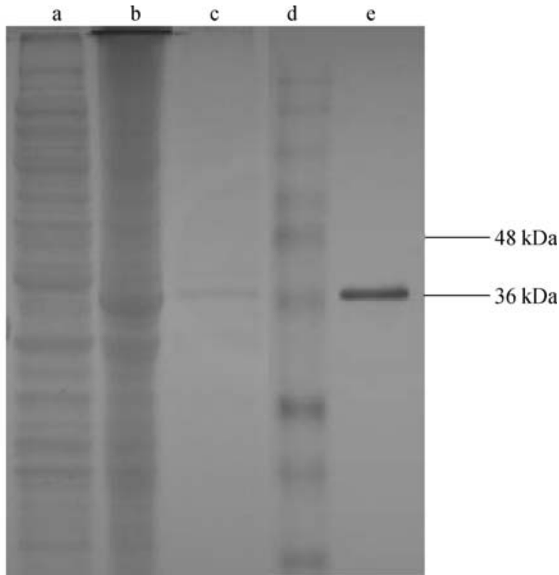

Fig. 2 SDS-PAGE of a lysate of *E. coli* expressing recombinant MacSe
Note: Lanes a–e represent *E. coli* lysate, *E. coli* containing MacSe, MacSe in eluate following nickel exchange chromatography, molecular ladder and immunoblot showing reaction of MacSe with convalescent horse serum, respectively.

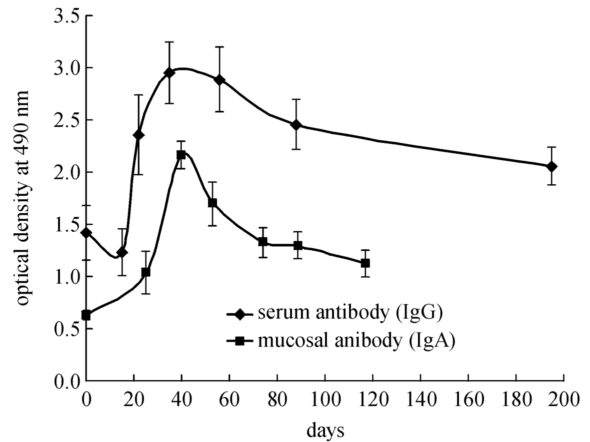

Fig. 3 Serum antibody and mucosal IgA response to MacSe
Note: The data obtained from 6 horses before, during and following commingling exposure to an *S. equi* ssp. *equi* infected horse on day 0.

3.3 Reactivity of recombinant MacSe with antiserum to Mac8345 of *S. pyogenes*

A mean OD of 2.9 was measured for the reactivity of rabbit antiserum (1∶100) to Mac8435 of *S. pyogenes* with recombinant MacSe in an ELISA. The OD value for normal rabbit serum was 0.1.

3.4 Convalescent antibody in sera and nasal washes

Specific antibody levels in sera (IgG) and nasal washes (IgA) to MacSe are shown in Fig. 3. MacSe specific IgG in serum peaked at about 30 d and then decreased gradually over the following 5 months. Specific IgA in nasal washes peaked between 40–45 d, and declined gradually over the following 80 d.

3.5 Binding of biotinylated MacSe to C3 and PMNs, endopeptidase activity of MacSe

Binding of MacSe to C3 on *S. equi* ssp. *equi* is shown in Fig. 4. Evidence for direct binding of MacSe to PMNs was also obtained. Figs. 5(a) and 5(c) display the granularity as a function of the fluorescence intensity of equine

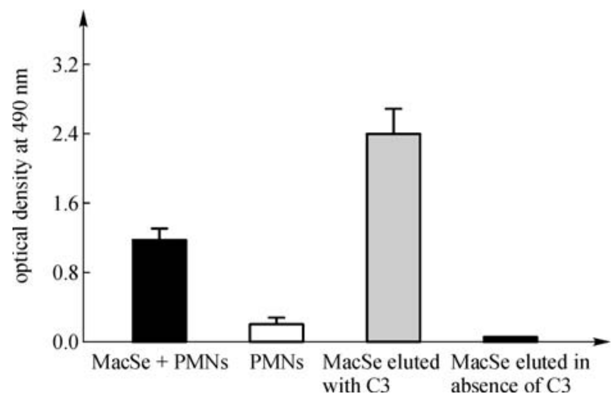

Fig. 4 Binding of biotinylated MacSe to equine PMNs and to C3
Note: Binding of biotinylated MacSe to PMNs was measured after incubation of a suspension of equine PMNs with MacSe. The control showed the value for sonicated PMNs in the absence of MacSe. Log phase *S. equi* ssp. *equi* (CF32, Irish1 and E23) were incubated with fresh horse naïve serum, washed and incubated with biotinylated MacSe for 1 h. One mol·L^{-1} hydroxylamine was added to the cells after washing to elute bound C3. ELISA was performed on the eluted supernatant to measure associated MacSe using HRP-avidin. The control showed an amount of MacSe recovered when fresh serum (C3) was omitted.

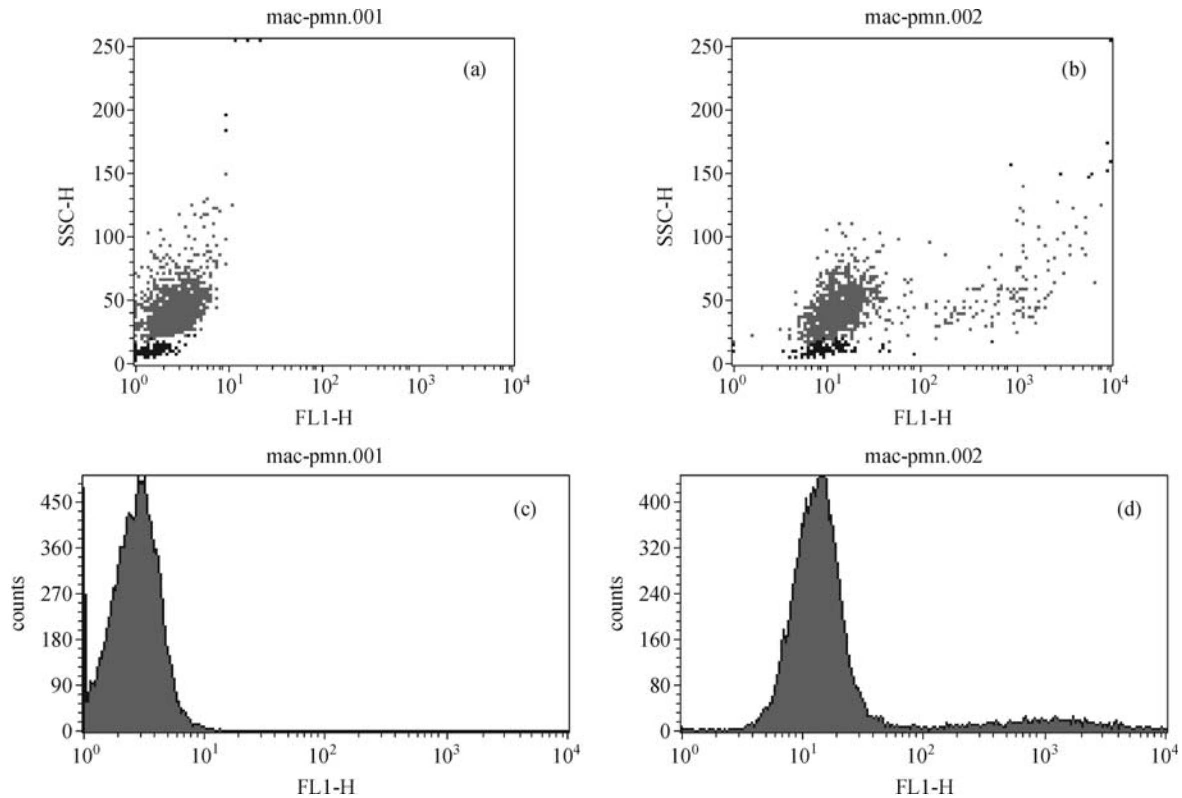

Fig. 5 Fluorescent staining and flow cytometry analysis of binding of biotinylated MacSe to equine neutrophils

Note: (a) and (c) represent neutrophils incubated with FITC-conjugated streptavidin. (b) and (d) represent neutrophils incubated with biotinylated MacSe for 1 h, washed, and incubated with FITC-conjugated streptavidin for 30 min. Biotinylated MacSe binding to neutrophils results in an increase in fluorescence ((b) and (d)) compared to control (a) and (c). Data representatives of three separate experiments are shown.

peripheral blood leukocytes after incubation with streptavidin-FITC without prior incubation with the biotinylated MacSe protein. Figs. 5(b) and 5(d) show that MacSe specifically bound an equine neutrophil subpopulation. However, all the cells were slightly stained, which suggests that they were all slightly bound by MacSe. During this assay, only 22.6% neutrophils were stained although several attempts to increase the protein concentration or decrease the cell number were made. We found that MacSe had no endopeptidase activity for equine IgGb, IgA, and IgM.

3.6 Levels of MacSe in culture supernatant *S. equi* ssp. *equi*

Release into culture supernatants of *S. equi* ssp. *equi* was not detected until 8 to 9 h after inoculation at onset of the log phase (Fig. 6) and ceased at mid-log phase.

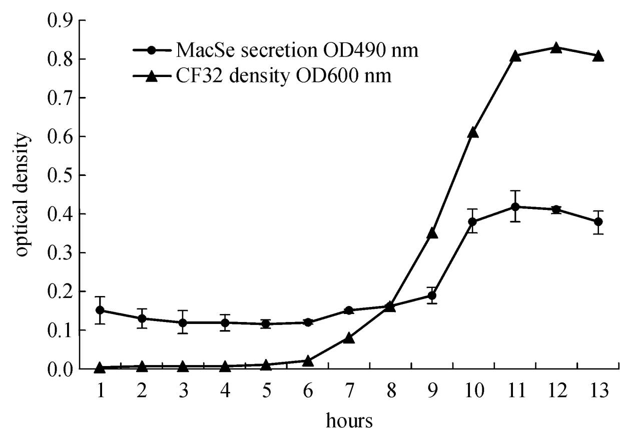

Fig. 6 Relation of secretion of MacSe into culture supernatant (OD490 nm) with the growth of *S. equi* ssp. *equi* CF32 (OD600 nm) *in vitro*

Note: MacSe in the supernatant was measured by ELISA with rabbit antiserum to recombinant MacSe.

3.7 Effect of MacSe on phagocytosis of *S. equi* by equine PMNs

Equine PMNs incubated for 60 min with opsonized *S. equi* ssp. *equi* CF32, e23 and 35683 in the presence of varying amounts of recombinant MacSe showed dose-dependent reduction in phagocytic/bactericidal activity (Fig. 7).

MacSe specific rabbit antiserum neutralized the inhibitory effect of 5 μg·mL^{-1} MacSe. *S. equi* spp. *equi* in the experiment were opsonized for 30 minutes at 37°C with SeM specific rabbit serum (1∶50).

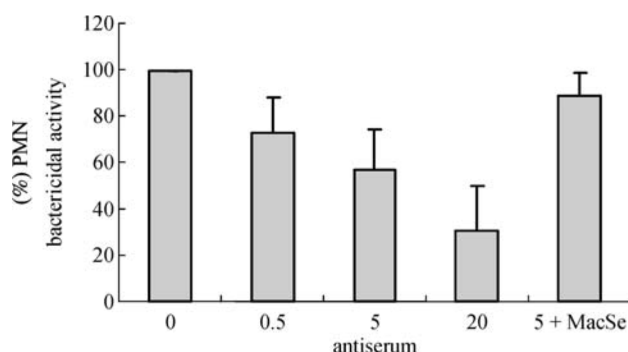

Fig. 7 Dose-dependent inhibition of PMN phagocytic activity by MacSe (μg)

Note: PMNs were incubated for 60 min separately with preopsonized *S. equi* ssp. *equi* CF32, e23 and 35683 in the presence of 0.5, 5, 20 μg·mL^{-1} of MacSe. Bactericidal activities of PMNs for each treatment (after 60 min) were expressed as percentages of CFU of *S. equi* ssp. *equi* at 60 minutes at 0 μg·mL^{-1} of MacSe. The opsonin was SeM specific rabbit antiserum.

4 Discussion

Surface components that contribute to its antiphagocytic behavior are the constitutively expressed hyaluronic acid capsule, the fibrinogen binding SeM (Harrington et al., 2002). These factors combined account for the very long chains of *S. equi* ssp. *equi* typically seen in acutely infected lymph nodes despite the presence of heavy infiltrates of neutrophils. However, serum antibodies to SeM do not protect ponies against commingling challenge and so it may be concluded that other antiphagocytic factors are involved in subversion of the innate immune response. Our study provides evidence that MacSe is an additional immunogenic protein secreted by *S. equi* that contributes to resistance to phagocytosis.

MacSe is an allele of mac complex II of *S. pyogenes*. Its amino acid sequence is 62.4% and 67.5% identical to the amino acid sequences of the prototype Mac sequences 5005 and 8345 *S. pyogenes* of complexes I and II respectively. The central variable region of MacSe aa89 to 300 is more similar to Mac8345 than to Mac5005 (Lei et al., 2001; Agniswamy et al., 2004). These workers also concluded that the closer relationship of MacSe to complex II variants is an indication that complex II variants are more ancient than complex I. MacSe contains a single RGD (aa244-247) that, by analogy with Mac5005 and Mac8345, may serve as a binding site for integrins 2vp3 and 211bβ3. It is of interest that the Mac proteins of *S. pyogenes* were designated as such on the basis of a region of partial homology they shared with the 2-subunit of Mac-1 (CD11b or complement receptor Type 3), a leukocyte P2 integrin found on neutrophils (Corbi et al., 1988). However, the mechanistic significance of this homology has not been elucidated. The data for *S. pyogenes* indicates that Mac disrupts the interaction between human CD16 and immune complexes (Lei et al., 2001) although Mac sequence mimicry is shared with CD11b. Since the latter and CD16 are physically associated, binding of Mac to CD16 may change the conformation/function of CD11b. As a possible explanation it has been suggested that the ability of Mac to bind CD16 is mediated by its β propeller homology to CD11b (Lei et al., 2002). We found that the nucleotide homology between the MacSe and part sequence available of horse CD11b is less than 40%, and amino acid homology is about 10%. This homology significance is still to be clarified. During our study both ELISA and cytometry analysis showed MacSe could be bound to neutrophils, which suggests potential significance of the homology.

Rapid phagocytosis happened after opsonization with SeM specific rabbit serum not with none-immune serum. Because the non-immune serum was ineffective at mediating opsonization, it is probable that sera containing the specific antibody would promote the complement deposition on the bacterial surface and mediate binding and ingestion by PMNs. MacSe could decrease the phagocytosis of *S. equi* ssp. *equi* and this effect was abrogated by anti-MacSe antibody. Phagocytosis was not completely inhibited by MacSe, likely due to ingestion facilitated by bacterial surface components other than those targeted by MacSe. All suggested that MacSe could block phagocytosis due to its disruption of an antibody and/or complement mediated interaction with receptors on the PMN cell surface. The binding of MacSe to C3 showed that MacSe does have affinity to C3, which is a critical element of innate immunity. In both the classical and alternate pathway, C3 participates in immune adherence and enhances phagocytosis. In this study we first showed that MacSe could bind to C3, possibly implicating that MacSe may affect the cascade of classical or alternative pathways through C3. One possible explanation of antiphagocytic activity of MacSe is that MacSe could be bound to C3, and then C3 or iC3b could not be bound to CD11b/CD18 and then inactivate the PMNs. This idea was supported by Nilsson et al. (2005) that ligation of CD11b/CD18 by bacteria-bound iC3b is necessary for inducing a neutrophil response leading to elimination of *S. pyogenes* in immune human serum.

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