Short Communication

Vaccination with streptococcal extracellular cysteine protease protects mice against lethal challenge with heterologous group A streptococci

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Running Title: Vaccination against group A streptococci

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[Abstract]

Virtually all clinical isolates of group A streptococci secrete a highly conserved extracellular cysteine protease that cleaves human fibronectin and vitronectin, and converts IL-1β precursor to biologically active IL-1β. Based on the high degree of gene conservation within the species and its role in host pathogenicity, it was postulated that antibodies to the cysteine protease would confer protective immunity against *S. pyogenes* infection. To test this hypothesis, Swiss CD1 mice that were intraperitoneally administered either saline, rabbit IgG, or IgG from rabbits immunized with the protease, were challenged with a highly virulent (minimum lethal dose ~ 10 cfu) clinical isolate of *S. pyogenes* expressing a heterologous cysteine protease. The results indicate that mice administered IgG from rabbits immunized with purified cysteine protease had significantly enhanced survival when compared with mice given either non-specific rabbit IgG or saline (log rank test; χ^2; P < 0.001). Moreover, mice actively immunized with the cysteine protease had a significantly longer time to death than the control group (log rank test; χ^2; P < 0.01). The results show that the cysteine protease elicits non-type-specific immunity to lethal challenge with heterologous *S. pyogenes*.

Introduction

Streptococcus pyogenes (group A *Streptococcus*) is a major agent of human morbidity worldwide. The pathogen causes a heterogeneous array of diseases, including pharyngitis, pneumonia, acute rheumatic fever, poststreptococcal glomerulonephritis, cellulitis, bacteremia, and meningitis.[1] Recent outbreaks of acute rheumatic fever,[1] and an increase in episodes of toxic-shock-like syndrome,[2,3] have emphasized the lack of an efficacious vaccine against this human pathogenic bacterium.

The primary focus of contemporary *S. pyogenes* immunoprophylaxis research has been M protein, an α-helical coiled molecule that projects outward from the bacterial cell wall and elicits type-specific immunity.[4,5] However, several problems have hindered development of an M-protein based vaccine. First, there is extensive variation in this molecule as demonstrated by the existence of greater than 80 serologic types. Moreover, there is considerable concern that an M protein vaccine might evoke human autoimmune-mediated disease because of sharing of epitopes with host tissues.[6-8] As a consequence, several groups have begun to investigate other vaccine candidate molecules,[9-13] stimulated in part by the observation that non-type-specific protection can be achieved in animal models of infection,[9,14] and evidence that nonopsonic antibody can mediate protection.[9]

Considerable indirect evidence suggests that an extracellular cysteine protease, or its proteolytically inactive precursor, participates in the pathogenesis of *S. pyogenes* disease episodes.[15-22] For example, the cysteine protease cleaves inactive human interleukin-1β precursor (pIL-1β) to produce biologically active IL-1β, a major cytokine mediating inflammation and shock.[15] Patients with fatal group A

Streptococcus infections have lower acute-phase serum antibody levels to this molecule than do patients with less severe infections, an observation consistent with a protective role for anti-protease serum antibody.[16] Individuals with streptococcal infections seroconvert to protease or its zymogen, which means that the molecule is expressed in vivo.[17] Poon-King et al.[18] recently reported that a streptococcal extracellular product historically referred to as nephritis-associated protein (NSAP)[19] is identical to all or part of the streptococcal protease precursor. Purified cysteine protease cleaves fibronectin, and rapidly degrades vitronectin, two human extracellular matrix proteins.[20] The protease also cleaves fibronectin from human umbilical vein endothelial cells (HUVEC) grown in culture, and rapidly causes a striking cytopathic effect when incubated with these cells.[20] These results show that the cysteine protease can disrupt the integrity of several host components, and therefore may influence host-parasite interactions. Talkington et al.[21] have reported that there is a statistically-significant association of extracellular protease production and soft tissue necrosis among invasive streptococcal strains recovered from patients in the United States. Protease activity was also statistically associated with occurrence of shock and organ involvement. Moreover, with a single exception, all organisms have the cysteine protease structural gene (*speB*).[20,22] This gene found to be highly conserved in a sample of 67 *S. pyogenes* strains expressing 39 distinct M protein serotypes and 5 provisional serologic types, a sample representing the breadth of genetic diversity present in the species.[20] In addition, virtually all strains were shown to produce protease.[20]

Taken together, the data suggested to us that further exploration of an immunoprophylaxis role for the cysteine protease was warranted. The goal of the present study was to directly test the hypothesis that serum antibody directed against the cysteine protease confers protection against lethal challenge with *S. pyogenes* in a

mouse model of invasive disease. We tested this hypothesis with both passive administration of polyclonal rabbit antibody raised against purified protease and active mouse immunization experiments. The results demonstrate that antibodies directed against this enzyme provide protection against lethal group A streptococcal infection.

Results

Minimum lethal dose

The minimum lethal dose (MLD), the highest dilution of *S. pyogenes* strain MGAS 315 at and below which there were no surviving mice, was approximately 10 organisms per inoculum, indicating that the strain MGAS 315 is unusually virulent to mice when administered via the intraperitoneal route. Pure cultures of group A streptococci were recovered from the heart blood of all mice that died after bacterial challenge.

Passive immunization with rabbit antiserum directed against purified cysteine protease

The results (Fig. 1) show that passive immunization with rabbit antibody directed against gel-purified denatured cysteine protease confers significant protection against challenge with highly virulent *S. pyogenes* strain MGAS 315 when compared with control animals given phosphate buffered saline (PBS) or pre-immune serum controls (log rank test; χ^2; P < 0.001). The protection afforded by passively administered antiserum was considerably higher during earlier (< 65 h) rather than later time points (Fig. 1). These results are especially significant because the

experiment was specifically designed to minimize the likelihood of demonstrating protection since; (i) the rabbit antibody was raised against gel-purified denatured cysteine protease and not native zymogen or active protease forms, (ii) in addition to the protease, the challenge strain is known to express pyrogenic exotoxin A (SPEA) and the recently described streptococcal superantigen, SSA,[23] and (iii) the cysteine protease precursor made by the challenge strain (SPEB1) differs from the protease precursor variant against which the antiserum was raised (SPEB4) at two amino acid positions (Ala <-> Val at position 111 and Ser <-> Gly at position 308; Fig. 2).[20]

Protection induced by vaccination with purified cysteine protease

The results (Fig. 3) show that intraperitoneal immunization with purified streptococcal cysteine protease also conferred significant protection (log rank test; χ^2; P < 0.01) against lethal challenge with the highly virulent *S. pyogenes* isolate MGAS 315. It is noteworthy that immunization with the cysteine protease also conferred significant protection against *S. pyogenes*-induced early mortality in mice. For example, all 10 mice in the control group were dead by 28 h post challenge, but only 4 of 9 mice died in the protease immunized group (difference in proportions; z; P < 0.003). Moreover, at the termination of the experiment at 120 h, 2 of 9 mice in the protease-treated but none of 10 mice in the control group survived (difference in proportions; z; P < 0.059). Thus, similar to the result observed with mice given immune rabbit serum, active immunization with the streptococcal cysteine protease conferred significant protection against lethal group A streptococcal infection.

Discussion

Although antigens eliciting protective immunity against group A streptococcal infection were first identified more than seven decades ago,[24] progress in the development of vaccines against this human pathogenic bacterium has been slow. Early work[25] suggested that the group A streptococcal surface M protein elicited protective opsonic antibodies, and since then, most streptococcal immunoprophylaxis research has been focused on this molecule. However, production of an M protein based human vaccine have been thwarted by two major problems. First, antibodies to M proteins are known to cross react with various host tissues.[5] Second, the greater than 80 identified M protein types elicit a predominantly type-specific immunity. Hence, an effective M protein based vaccine would need to be highly polyvalent or directed against yet unidentified conserved pan-protective M protein epitopes.

In contrast to the extensive efforts directed at developing an M protein based vaccine, there have been relatively few attempts to identify streptococcal antigens that elicit non-type-specific immunity. However, several groups have recently demonstrated that antibodies to molecules other than M proteins may provide protection against *S. pyogenes* infection. Chappell and Stuart[12] found that intraperitoneal inoculation of washed bacterial cultures of an M-negative isolate protected mice against lethal challenge with *S. pyogenes* strains expressing M3, M18, or type 28 surface molecules. They attributed this non-type-specific immunity to the presence of antibodies against three proteins of M_r 32, 43 and 46 kDa. Stjernquist-Desatnik *et al.*[10] showed that intranasal vaccination with heat-killed isolates of M-negative strains of *S. pyogenes* protected against intranasal challenge with an isolate expressing serotype M50, and Rotta *et al.*[26] demonstrated heterologous protective

immunity in mice immunized via the intraperitoneal route with streptococcal cell wall preparations or material enriched in peptidoglycan, a group A streptococcal cell wall component. O'Connor and coworkers[11] have suggested that neutralizing antibodies directed against the streptococcal cell surface enzyme C5a peptidase may provide protection against streptococcal colonization, but have not directly tested this hypothesis. A recent study,[13] suggested that organisms pretreated with antibodies to the group A streptococcal surface molecule lipoteichoic acid (LTA) are significantly attenuated in a mouse model of infection. However, due to the lack of inclusion of a non-specific antibody control in that study,[13] it is not possible to determine if the protective effect of the antibodies was due to interaction with LTA, or is merely due to the non-specific binding of immunoglobulin to the bacterial surface.

There is a growing body of evidence suggesting that the streptococcal extracellular cysteine protease is an important virulence factor.[15-22] Our results clearly demonstrate that antibodies raised against the purified enzyme provide significant protection against heterologous lethal group A streptococcal infections in mice. Although the exact reason(s) for delay in onset of mortality in mice that were administered anti-protease antibody are not known, one of the hypotheses that can account for this observation is that passively administered rabbit antibody may have a short half-life in mouse circulation, hence, a decrease in the circulating levels of specific antibodies with the passage of time may account for a delay in onset of mortality. A second, but not mutually exclusive, hypothesis is that passively administered antiserum was not of sufficiently high titer to neutralize all of the organisms inoculated.

Although our studies were performed with a mouse model of infection, the relevance of these results to human infections is supported by the observation that

patients with lower levels of acute phase serum antibody to the streptococcal protease precursor are more likely to die or do poorly than are patients with high levels of anti-protease precursor antibody.[16] In this context, the results of our study suggest that administration of anti-protease antibody or a physiologically compatible protease inhibitor to patients with severe life-threatening illnesses may be of therapeutic value. However, additional studies are needed, possibly with primate models of infection. Since our investigation was not designed to address the specific contribution of local and systemic immune response to the protease in providing protection against lethal challenge, we are presently examining this question. In addition, it will be of considerable interest to examine the role of antibodies specific to the protease precursor[27] in providing protection against *S. pyogenes* infection.

Methods

Bacterial strains

The bacterial strains used in this study have been described.[20] *S. pyogenes* isolate MGAS 1719 is identical to strain B220 and was obtained from Dr. K. H. Johnston, Louisiana State University, New Orleans, LA. This isolate expresses T8 serotype, and has the *speB7* allele,[20] and was used as the source strain for cysteine protease purification. Isolate MGAS 315 was recovered in the 1980s from a patient with toxic-shock-like syndrome. The organism is electrophoretic type (ET) 2, expresses serotype M3 protein, and was used as the challenge strain. This isolate also synthesizes SPEA and SSA.

Purification of streptococcal cysteine protease

Streptococcal cysteine protease was purified from strain MGAS 1719 culture supernatants with a combination of ultrafiltration and dye-ligand affinity chromatography.[15,20] SDS-polyacrylamide gel electrophoresis and coomassie blue staining of the resulting proteolytically active material showed a single major band of $M_r \sim 30$ kDa.[15] The purified cysteine protease preparation does not react with rabbit antiserum raised against acid extracts of M serotype 1 or 3, or type T8 *S. pyogenes* isolates by either western immunoblot or ELISA, but reacted strongly with rabbit antibodies raised against the streptococcal cysteine protease (Fig. 4).

Calculation of bacterial minimum lethal dose

The minimum lethal dose (MLD) of bacteria was calculated for isolate MGAS 315. Bacteria were grown in brain heart infusion broth (Difco, MI) for 12 h at 37 C, the A_{600} was adjusted to 0.7 (representing $\sim 10^8$ cfu/ml) with sterile BHI broth, and 0.1 ml of 10^0 through 10^{-7} dilutions of bacteria made in sterile broth were injected intraperitoneally to each of five male 22-24 g CD-1 outbred mice (Charles River, MA). The bacterial suspensions were tested for purity before and after the inoculations, and the number of cfu injected per mouse was verified by colony counts. The mice were monitored for a period of 5 d post-inoculation and surviving mice were euthanized with methoxyflurane. Heart blood was collected for bacterial isolation from the mice that died or were sacrificed. The MLD was determined to be the highest dilution at or below which none of the mice in the group survived.

Passive immunization of mice

The preparation of rabbit antiserum against streptococcal cysteine protease has been described.[20] Male 22-24 g CD-1 outbred mice were intraperitoneally inoculated with 0.1 ml PBS ($n = 6$), 1 mg immune rabbit IgG in PBS ($n = 9$), or 1 mg pre-immune rabbit IgG in PBS.($n = 9$) and challenged with the MLD of strain MGAS 315, 30 min after the administration of the treatment. The mice were monitored at 3 h intervals and mortality was recorded. Heart blood was collected from dead mice and plated onto BHI agar, and incubated for 48 h at 37 C in 5% CO_2. Kaplan-Meier survival curves were plotted and the logrank test[28] was employed to test for statistical differences in survival.

Active immunization of mice

Male Swiss CD1 outbred mice were inoculated with either PBS ($n = 10$) or 20 µg of purified streptococcal cysteine protease in PBS ($n = 9$) subcutaneously on day 1, followed with intraperitoneal inoculations of the same treatments at days 7, 14, 21, 42, 50, 57, 63, and 79 for a total of nine immunizations. Serum antibody levels to the cysteine protease were checked at days 29, 71, and 84 by ELISA, and the mice were challenged with strain MGAS 315 on day 93, two weeks after the last immunization. The mice were monitored at 3 h intervals, mortality recorded, and Kaplan-Meier survival curves[28] plotted and analyzed as described above.

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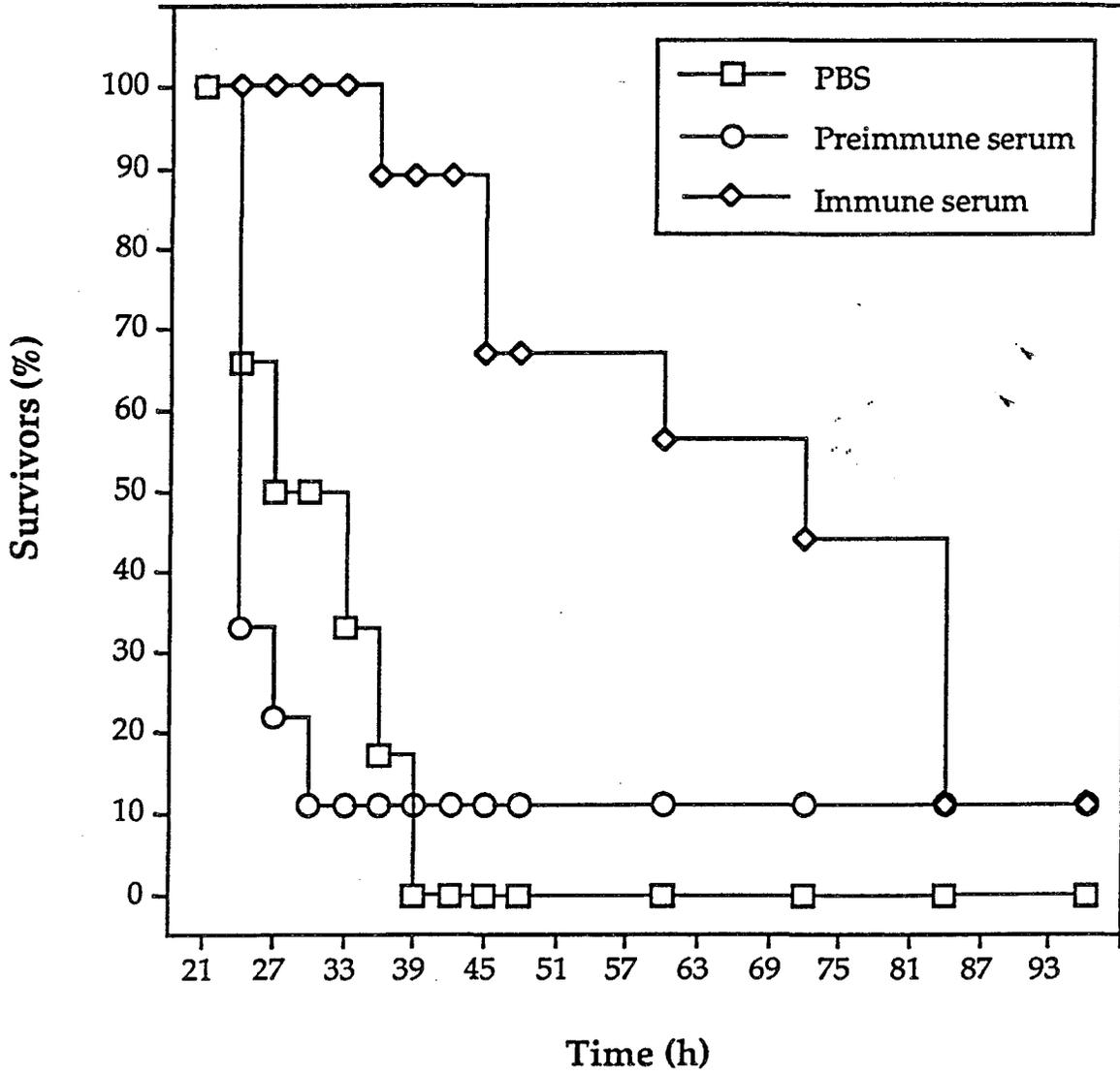28. Pocock SJ. Clinical Trials: a practical approach. New York: John Wiley, 1983: 224-5.
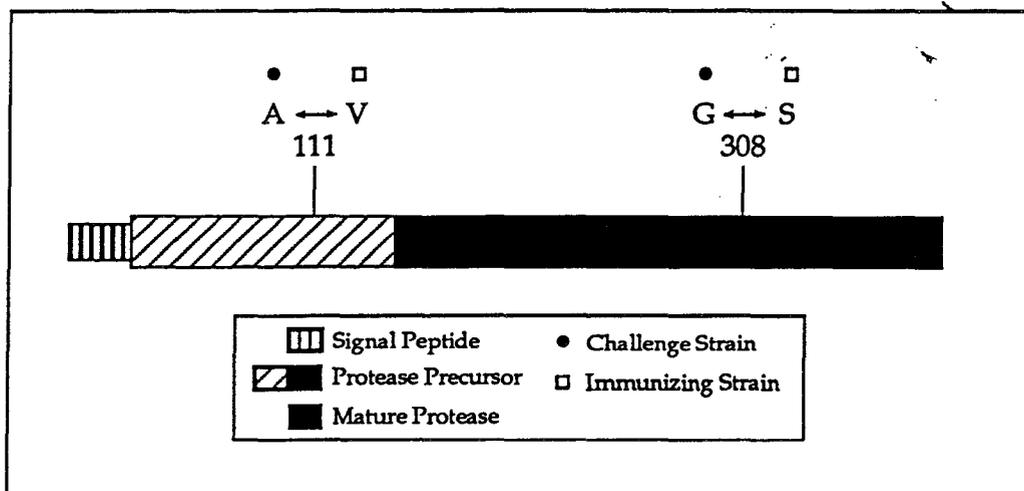
[Figure Legends]

Fig. 1. Passive administration of anti-protease antibody protects mice against lethal challenge with heterologous *S. pyogenes*. Intraperitoneal administration of rabbit antibody directed against streptococcal cysteine protease confers significant protection (log rank test; χ^2; P < 0.001) against lethal challenge with the highly virulent *S. pyogenes* isolate MGAS 315 when compared with control animals that were given PBS or rabbit pre-immune serum.
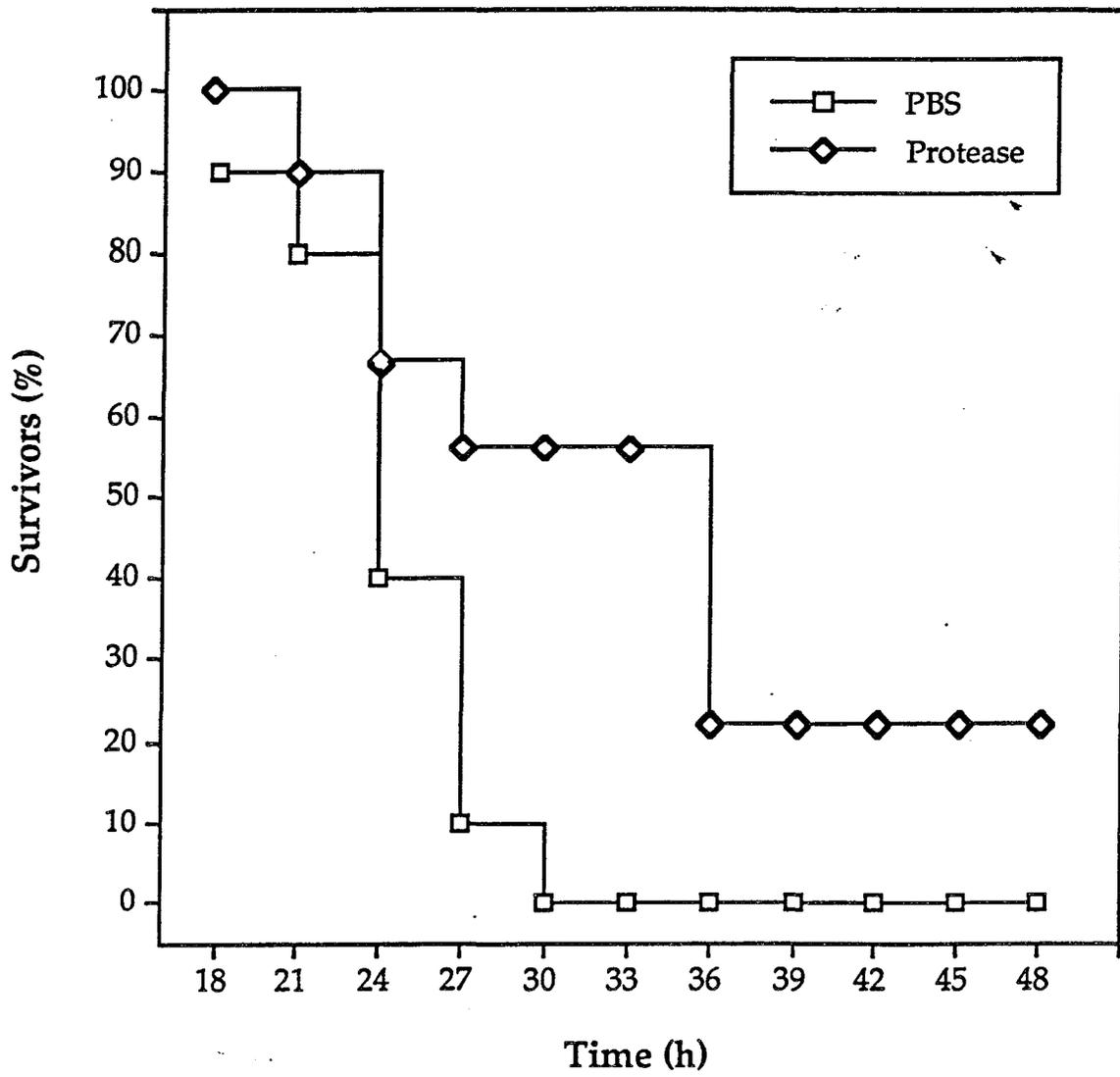
Fig. 2. Schematic representation of amino acid substitutions in the streptococcal cysteine protease and protease precursor in the challenge strain (•) and the protease to which the antiserum was raised (□). A short peptide fragment of the protease surrounding amino acid 308 contains immunodominant epitopes recognized by mouse polyclonal and monoclonal antibodies (Kapur and Musser, manuscript in preparation). The single letter amino acid abbreviations are A, Ala; V, Val; G, Gly; S, Ser.

Fig. 3. Active immunization of mice with the streptococcal cysteine protease protects against lethal challenge with heterologous *S. pyogenes*. The data show that intraperitoneal immunization with purified streptococcal cysteine protease conferred significant protection (log rank test; χ^2; P < 0.01) against lethal challenge with the highly virulent *S. pyogenes* isolate MGAS 315.

Fig. 4. Rabbit serum to streptococcal M3 protein (lane 1), T8 protein (lane 2), or purified IgG from preimmune rabbits (lane 3), does not react with the streptococcal protease. In contrast, purified IgG from rabbits immunized with the protease reacts specifically with the $M_r \sim 30$ kDa streptococcal extracellular cysteine protease used in active immunization experiments (lane 4).

Signal Peptide   ● Challenge Strain

Protease Precursor   □ Immunizing Strain

Mature Protease