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Anomalous Immunogenic Properties of Serine Proteases

H. Y. Darani* & M. J. Doenhoff†

*Department of Parasitology, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran; and †School of Biology, University of Nottingham, University Park, Nottingham, UK

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Correspondence to: H. Y. Darani, Department of Parasitology, Cell and Molecular Research Center, Faculty of Medicine, Rahmatieh, Shahrekord University of Medical Sciences, Shahrekord, Iran. E-mail: h_yousofi@yahoo.com

Abstract

It has previously been shown that a ~27 kDa serine protease of *Schistosoma mansoni* larvae, the cercarial elastase (CE), was a poor immunogen in as much as it failed to induce an antibody response. The CE has a critical role in enabling schistosome larvae to penetrate the skin of their definitive hosts, so the apparently poor immunogenicity of this enzyme is clearly of interest. To understand its lack of immunogenicity better and in particular to determine whether it is related to its proteolytic activity, we have measured antibody responses of mice to three different serine proteases. Groups of mice were immunized with porcine pancreatic trypsin (TRY), chymotrypsin (CHY) or elastase (ELA) and the resulting antibody response compared with antibody responses to two non-protease antigens, chicken egg albumin (OVA) and *Schistosoma japonicum* glutathione S-transferase (GST), all being administered with alum as an adjuvant. Of 12 mice that were injected five times at 14 day intervals with TRY, only one produced antibody reactive with this enzyme in ELISA. Immunizations with CHY or ELA induced somewhat better antibody responses than TRY, but the responses to the first and second injections of these two proteases nevertheless seemed comparatively lower than the responses to GST. Induction of antibody responses by OVA and GST was not affected when TRY was injected concomitantly. Thus, the antibody response to one of the serine proteases used in this study, mammalian trypsin, was anomalous.

Introduction

A ~27 kDa serine protease secreted from the acetabular glands of *Schistosoma mansoni* cercariae, the cercarial elastase (CE), has a critical role enabling the schistosome larvae to penetrate the skin of their definitive hosts [1]. We have previously shown that antibodies against the CE were absent in sera from rabbits repeatedly infected percutaneously with large numbers of *S. mansoni* cercariae, although antibodies against other constituents of the larvae were present [2]. Furthermore, only a minority of inbred mice that had been immunized with purified CE had antibody responses to the enzyme detectable by Western immunobloting [2]. These results in experimental animals indicated that *S. mansoni* CE was relatively non-immunogenic. The observation that the enzyme could degrade both rabbit and human IgG [2] suggested that the low immunogenicity might be because of its proteolytic activity.

Investigations on antibody responsiveness to the CE in infected humans have given equivocal results. One study indicated that anti-CE antibodies could be used as an early marker of human exposure to schistosome infection [3], while in another anti-CE antibodies were not detectable in any sera from infected humans [4].

Some proteases have been used for vaccination, serodiagnosis or other purposes, thus indirectly indicating they are immunogenic [5–8], but there seems to have been no formal investigation of the immunogenicity of proteases. To determine whether the poor immunogenicity of the CE in experimental animals might be related to its proteolytic activity, we have compared the antibody responses of mice to three mammalian proteases, trypsin, elastase and chymotrypsin, with responses to two molecules without protease activity, ovalbumin and glutathione S-transferase.

Materials and methods

Groups of inbred male CBA mice, approximately 6 weeks-old, were immunized with three proteases: porcine pancreatic trypsin (TRY), porcine elastase (ELA)

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or bovine pancreatic chymotrypsin (CHY; all purchased from Sigma/Aldrich, Poole, UK); and two non-protease antigens, chicken egg ovalbumin (OVA; Sigma/Aldrich, Poole, UK) and *Schistosoma japonicum* glutathione S-transferase (GST; Sigma/Aldrich). Each mouse was primarily immunized by injection of 0.2 ml of a 10% (v/v) suspension of aluminium hydroxide adjuvant to which had been absorbed 20 μg of each of the proteins. Control mice were injected with alum alone. Each mouse was given further booster injections intraperitoneally at intervals of a fortnight, each injection containing 20 μg protein absorbed on alum. The mice were bled fortnightly, 2 weeks after every immunization and immediately before administration of a booster immunization.

Antisera specific for TRY were raised in adult New Zealand white rabbits by repeated fortnightly injections of 0.5 ml saline containing 10 mg purchased porcine TRY (Sigma) emulsified in Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the boosters. Rabbits were bled following the last booster and sera were then collected and stored at −20 °C. Serum from an intact normal adult New Zealand rabbit was used as a negative control serum.

Sera from immunized mice and also the rabbit positive and negative sera were tested for the presence of antibodies by ELISA as described by Fallon *et al.* [9]. Polystyrene microtitre plates were coated with the five different antigens diluted in coating buffer (pH 9.6) at 37 °C for 1 h followed by overnight at 4 °C. The coating concentration for each antigen was determined by performing a checkerboard titration.

The antigen-coated plates were washed three times in washing buffer, after which the plates were incubated for 1 h with blocking buffer (100 μl per well) and washed again three times with washing buffer. The relevant test sera, diluted in incubation buffer, were added to the plates and incubated for 2 h at room temperature. Plates were washed and a relevant enzyme-conjugated anti-species immunoglobulin diluted in incubation buffer was added. The plates were incubated for 2 h and then washed. The substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in substrate buffer, activated by hydrogen peroxide, was added and the reaction allowed to develop prior to reading in a spectrophotometer at 414 nm.

The Mann–Whitney test was used for statistical analysis.

Results

To test the immunogenicity of porcine pancreatic trypsin (TRY), 11 mice were injected five times with this protease adsorbed on alum at intervals of a fortnight and a further six mice were injected with alum alone on the same days as the control mice. Fig. 1 shows that by the end of the five-injection, 10-week immunization schedule, 10 of the 11 mice had produced no antibodies against TRY. Only one exceptional animal responded with antibody production after the second and subsequent injections of the protease.

The second experiment was designed to test whether the presence of TRY would affect the antibody responses to non-protease antigens when the former was present in a mixture with *S. japonicum* GST and/or OVA. The experiment was performed on 36 age-matched male CBA mice segregated into six groups, each group being immunized as follows: group 1, immunized with TRY alone; group 2, immunized with OVA alone; group 3, immunized with GST alone; group 4, immunized with both GST and OVA; group 5, immunized with GST, OVA and TRY.

As shown in Fig. 2, mice immunized with TRY, whether alone or together with GST and OVA, all failed to produce anti-TRY antibodies after the first and second immunizations. After the third injection, of the 12 mice immunized with TRY, only one had a higher than baseline antibody response, with an ELISA OD value of 0.6.

By contrast, the three groups of mice immunized with GST, whether alone or in the presence of OVA, or of both OVA and TRY all started to produce antibodies after the first injection and antibody levels in these increased markedly after the second injection (Fig. 3).

Titres of anti-OVA antibodies in the mice immunized with this protein were barely above baseline after the first immunization, but were substantial after the second and third injections (Fig. 4).

To investigate whether poor immunogenicity was a characteristic of well-known serine proteases other than TRY, groups of mice were immunized with either chymotrypsin (CHY) or elastase (ELA). Antibody responses to CHY and ELA were measured by ELISA in separate

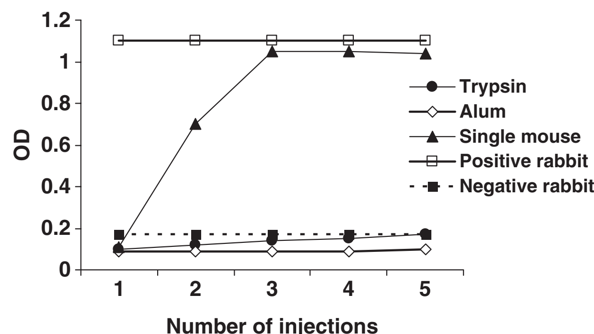

Figure 1 ELISA of antibodies to porcine pancreatic trypsin (TRY; coated on the plate) and probed with the sera of mice injected with trypsin on alum (•) or alum alone (◇), following the first (1), second (2), third (3), fourth (4) and fifth (5) injections. Lines (□) and (■) are positive and negative control rabbit sera respectively. ◀ = a single 'responder' TRY-injected mouse.).

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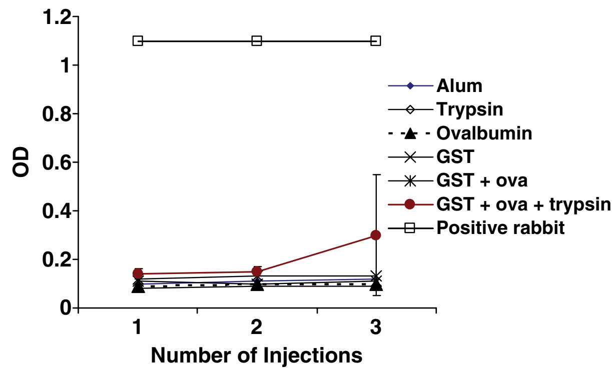

Figure 2 ELISA of antibodies to trypsin (coated on the plate) and probed with the sera of mice injected with alum alone (♦), trypsin (◇), ovalbumin, (OVA; (◀) GST (x), OVA + GST (*), OVA + GST + trypsin (•) following the first (1), second (2) and third (3) injections. □ = rabbit positive control serum. Standard deviation in OVA + GST + trypsin-injected mice (•) was 0.01, 0.01 and 0.25 for the first, second and third injections respectively.)

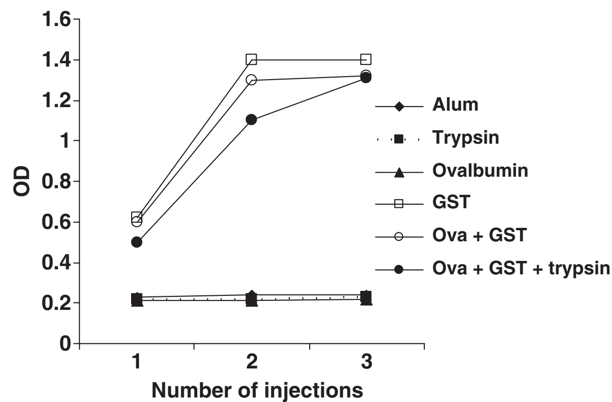

Figure 3 ELISA of antibodies to GST (coated on the plate) and probed with the sera of mice injected with alum alone (♦), trypsin (■), ovalbumin (◀), (GST (□), OVA + GST (○) and OVA + GST + trypsin (•) following the first (1), second (2) and third (3) injections. The difference in antibody titre between the alum alone-injected mice and the three groups of GST-injected mice was statistically significant ($P < 0.001$).

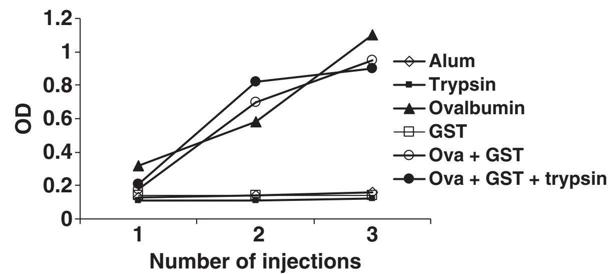

Figure 4 ELISA of antibodies to ovalbumin (coated on the plate) and probed with the sera of mice injected with alum alone (◇), trypsin (■), ovalbumin (◀), GST (□), OVA + GST (○) and OVA + GST + trypsin (•) following the first (1), second (2) and third (3) injections. The difference in the antibody titre between the alum alone-injected group and the OVA-injected groups was statistically significant ($P < 0.001$).

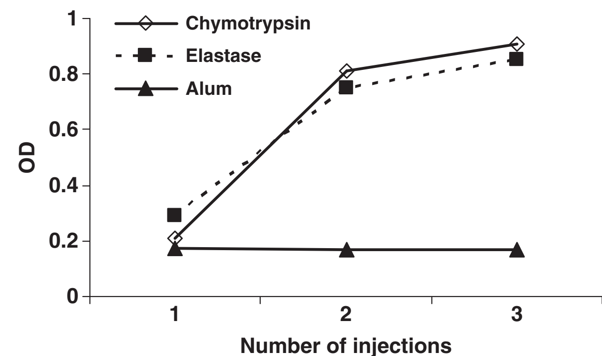

Figure 5 ELISA of antibodies to elastase (coated on the plate) and probed with the sera of mice injected with elastase on alum (■) or alum alone (◀) and ELISA of antibodies to chymotrypsin (coated in the plate) and probed with the sera of mice injected with chymotrypsin on alum (◇) or alum alone (◀), following the first (1), second (2) and third (3) injections.

microtitre plates coated with one of these two enzymes. The results are given in Fig. 5. After the first injections of each of these enzymes, the antibody responses were barely above base-line. However, antibody levels to both CHY and ELA increased in response to the respective second injections.

Discussion

This study was performed to test whether the apparent lack of immunogenicity of a serine protease of *S. mansoni* cercariae [2] is associated with the potent proteolytic activity of the molecule. To this end, antibody responses of mice to three mammalian proteases, trypsin and chymotrypsin (both from porcine pancreas) and bovine pancreatic elastase, were measured in ELISA and compared with antibody responses to two other proteins, *S. japonicum* glutathione S-transferase and chicken egg albumin (ovalbumin).

After repeated immunizations with TRY (five in the first experiment, three in the second), only two out of 24 mice produced antibodies against this serine protease. Mice produced better antibody responses to CHY and ELA and to two non-protease proteins GST and OVA, than to TRY. The antibody responses to GST and OVA were however not affected when TRY was present in immunizing mixtures of these three antigens.

In agreement with the results of this study, it has been shown that the schistosome cercarial elastase did not induce an apparent antibody response in infected patients [4]. In another investigation, it was shown that purified serine proteases from *Lucilia cupina* larvae failed to induce

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a strong immune response in ELISA [10] and in research aimed at developing an adenovirus protease as a vaccine, the env subunit boosted cellular, but not humoral immune responses [11]. However, other proteases such as some cysteine proteases were good immunogens when they were used for vaccination, serodiagnosis or other purposes [5–8].

It is not clear why some serine proteases are more immunogenic than others. Plasma serine protease inhibitors (serpins) might be involved as they control a wide variety of physiological functions involving proteases [12, 13], including blood coagulation, complement-activation and certain aspects of inflammatory responses and play a role in neutralization of proteolytic activity from exogenous sources. In this respect, they are believed to constitute a primitive defence mechanism against invading parasites [14–18]. When complexes between protease and serpin form they are likely to be rapidly removed from the circulation [17] and any heterologous protease involved in such complex formation may not be recognized as an immunogen. In a test of this hypothesis [16], rabbits repeatedly injected with complexes of trypsin and contrapsin (the latter being a serpin in mouse plasma) in complete Freund's adjuvant failed to make antibodies to the enzyme, indicating that the trypsin had seemingly lost its immunogenicity as a result of complexing with the serpin. This explanation is however incomplete as it does not indicate why CHY and ELA differed from TRY in being more immunogenic in the present study. Perhaps the immunogenicity of some proteases is better concealed in some enzyme-serpin complexes than in others, or serpins with appropriate specificity for reaction with CHY and ELA were absent.

Another, perhaps less likely explanation for lack of immunogenicity of some serine proteases may be their high degree of homology with the proteases of the immunized animal [14] with the result that they are immunologically indistinguishable from self. A third hypothesis relates to mechanisms of antigen processing and presentation in antigen-presenting cells (APC). To be recognized as immunogens proteins presented to the immune system must first be cleaved to small peptides by intracellular proteinases. These peptides, which have about 13–18 amino acid residues, then bind to major histocompatibility complex (MHC) molecules and are transported to the cell surface [19–24]. Eventually, the peptide-MHC complexes are recognized by helper T lymphocytes that stimulate inflammatory and antibody responses (reviewed by Goldberg and Rock [21]. However, when the immunogen is itself a protease, it may not be processed like a molecule without proteolytic activity because, for example, it degrades other proteins involved in the antigen-processing functions of APCs.

Finally, the lack of immunogenicity of some serine proteases may be connected to their ability to cleave the immunoglobulins, as has indeed been shown with respect to the schistosome CE [2, 25–28]. According to this hypothesis, poorly immunogenic proteases such as TRY and the *S. mansoni* cercarial elastase might cleave surface-bound immunoglobulins of B cells acting as APCs while other proteases that are more immunogenic may not have this ability.

In conclusion, results of this work showed that mammalian trypsin failed to induce an antibody response in most mice into which it was administered as an immunogen. Further work is needed to determine why trypsin has this anomalous immunological property and whether the phenomenon is relevant to evasion of immunity by some parasites and pathogens that depend on the activity of proteases for their survival.

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