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Studies on Epitope Recognition of the *Toxoplasma* Nucleoside Triphosphate Hydrolase

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ABSTRACT

The nucleoside triphosphate hydrolase (NTPase) of *Toxoplasma gondii* demonstrates an unusually high level of ATP hydrolysis, which, among the apicomplexan parasites, has only been observed in *T. gondii* and the closely related *Neospora caninum*. In *T. gondii*, NTPase has been shown to be highly expressed (constituting up to 8% of the tachyzoite protein) and is an immunodominant antigen in mice and humans. Two isoforms exist - NTPaseI and NTPaseII. NTPaseI demonstrates a 4.5 fold greater activity than NTPaseII with respect to ATP hydrolysis. Past studies suggest that only virulent strains possess the highly active NTPaseI isoform. We have recently identified a B cell epitope (aa 484-502) on the NTPase isoforms which, despite some cross reactivity, is differentially recognised by a naturally infected human serum sample. In this study we used competitive antigen ELISAs and have identified that this serum sample reacts specifically to the NTPaseI epitope, whilst the corresponding region on NTPaseII isoform is the less specific cross reactive epitope. These results are consistent with the hypothesis that this patient has been infected with a virulent strain of *T. gondii*.

INTRODUCTION

Although *T. gondii* is the only species in the genera *Toxoplasma*, pathogenicity studies in mice have identified the existence of mouse virulent and mouse avirulent strains (Ferguson and Hutchison 1981). The classification of virulent and avirulent strains based on both genetic and antigenic variability is becoming increasingly important, as recent studies have suggested a predominance of virulent strains associated with congenital toxoplasmosis, whilst avirulent

strains appear to be associated with toxoplasmic encephalitis (Cristina et al. 1995). Whilst genetic studies have identified numerous virulence/avirulence associated loci (reviewed by Johnson 1997), fewer markers have been identified at the protein level by immunological means. Using monoclonal antibodies, Gross et al. (1991), identified a 23 kDa membrane antigen which was only detected in virulent strains, whilst Bohne et al. (1993), identified a 27 kDa cytosolic antigen present only in avirulent strains. Meisel et al. (1996) also used a monoclonal antibody to identify an epitope sequence on the GRA4 antigen associated with virulence. Recently we described a B cell epitope sequence on the NTPase of *T. gondii* which is differentially recognised between the two NTPase isoforms by using synthetic peptides representing each isoform (aa 484-502) in an ELISA with a naturally infected human serum sample. This serum sample had a significantly greater reactivity to the NTPaseI peptide than to the NTPaseII peptide. This was of some interest because a previous study by Asai et. al. (1995) which identified the presence of the two isoforms (NTPaseI and NTPaseII), also found that only virulent strains possessed the NTPaseI gene, whilst all strains possessed the NTPaseII gene. Nakaar et. al. (1998) were able to confirm that only virulent strains expressed the NTPaseI gene product, whilst all strains tested expressed the NTPaseII gene product. In addition to this the isoforms are also distinguishable by the remarkable differences in substrate specificity and enzyme activity, with the NTPaseI isoform showing 4.5 fold higher activity in the hydrolysis of ATP (Asai et al. 1995). In fact, the region responsible for substrate binding and specificity, which was recently identified by Nakaar et al. (1998), also represents the area of greatest variability between the two isoforms (aa 488-499) and corresponds to the region in which we identified this differential antibody response.

The NTPase of *T. gondii* is a very abundant protein, as it constitutes up to 8% of the total tachyzoite protein and is also an immunodominant antigen in both mice and humans (Asai et al. 1987; 1992). Although the function of this highly active enzyme remains unknown, a recent study has demonstrated that repression of NTPase activity by antisense RNA leads to inhibition of parasite replication (Nakaar et al. 1997) and, as such, NTPase is essential to parasite survival. Reasons for the existence of the two isoforms also remain unknown, however, further immunological and functional studies of this important enzyme may shed new light on the function of NTPase isoforms. In this study we have further evaluated the reactivity of a naturally infected human serum sample to the differential epitope (aa 484-502) of NTPase by the use of competitive antigen ELISA.

MATERIALS AND METHODS

Antigens

Recombinant NTPaseI pGEX1λT was expressed in *E. coli* and purified from inclusion bodies as described previously (Johnson et al. 1999). 20 aa peptides

encompassing aa 484-502, NH2-CKAPMIVTGGGMLAAINTLK- OH on NTPaseI (NTPIpep) and NH2-CKAPMFITGREMLASIDTLK-OH on NTPaseII (NTPIIpep), were commercially manufactured (Chiron mimotopes). The residues CK at the n-terminus of these peptides are not part of the *T. gondii* NTPase sequence but were included for peptide stability.

Competitive antigen ELISA

Recombinant NTPaseI and peptide antigens were diluted to 5 μg/ml in 0.1 M carbonate/bicarbonate buffer pH 9.6 and 100 μl was used to coat each well of a flat bottom ELISA plate (Costar). The plates were incubated overnight at 4°C and then washed 4 times with PBS containing 0.03% Tween 20 (PBST). The plates were then blocked with 2.5% w/v skim milk powder in PBST and incubated for 1 hr at 37°C. The serum was diluted 1/100 in 1% w/v skim milk powder and preincubated at 37°C for 1 hr with 2 fold serial dilutions of the competing peptide. No peptide was added to the negative control serum. 100 μl of treated serum was then added to each well and the plates incubated and washed as before. Anti-human Ig conjugated to alkaline phosphatase (Dako) was diluted in 1% w/v skim milk powder and 100 μl added to each well. The plates were washed and incubated as before. One hundred microlitres of 1 mg/ml p-nitrophenyl phosphate substrate (Sigma) dissolved in 10% diethylamine buffer was added to each well and incubated at 22°C in the dark. The O.D. values were measured at 405 nm against a control well that had not received serum but did receive conjugate, thus allowing for non specific reactivity of the conjugated. Percent inhibition was determined by dividing the respective test wells (serum plus competing peptide) by the negative control well. This figure was multiplied by 100 to give percent inhibition.

Indirect ELISA

Peptide antigens were diluted to 2.5 μg/ml in 0.1 M carbonate/bicarbonate buffer pH 9.6. Flat bottom ELISA plates were coated with 100 μl of antigen solution per well, incubated overnight at 4°C and then washed 4 times with PBST. The coated wells were then blocked with 2.5% w/v skim milk powder in PBST and incubated at 37°C for 1 hr, followed by four washes with PBST. The serum was diluted in 1% w/v skim milk powder in PBST and 100 μl added to each respective well. The plates were incubated and washed as before. Anti-human IgM or IgG conjugated to alkaline phosphatase (Sigma) was diluted in 1% w/v skim milk powder and 100 μl added to each well. The plates were incubated and washed as before. One hundred microlitres of 1 mg/ml p-nitrophenyl phosphate substrate (Sigma) dissolved in 10% diethylamine buffer was added to each well and incubated at 22°C in the dark. The O.D. values were measured at 405 nm against a control well that had not received serum or conjugate.

RESULTS AND DISCUSSION

Competitive antigen ELISAs

When NTPIpep and NTPIIpep were used as competitor antigens against wells coated with recombinant NTPaseI in an ELISA, it was clearly evident that NTPIpep competes for antibody reactivity at much lower concentrations than NTPIIpep (Fig. 1). However both peptides yielded about the same level of inhibition at the highest concentration (125 μg/ml inhibitor). This is consistent with our previous findings that this patient's serum displays significantly greater antibody reactivity to the NTPI peptide than the NTPII peptide. In order to gain a more precise representation of this differential activity we used competitive antigen ELISA's using NTPIpep and NTPIIpep as the coated antigens. NTPIpep and NTPIIpep were then used as the competitor antigen against each of the coated antigens. This protocol allowed us to study the affinity of the serum for each of the peptides. When NTPIpep was used as the coated antigen (Fig. 2), it was observed that NTPIpep competed for antibody against itself at much lower

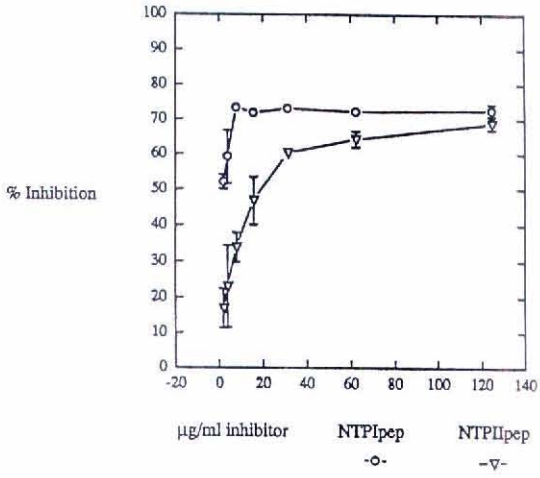

Figure 1. Competitive antigen ELISA. Plate coated with recombinant NTPaseI.

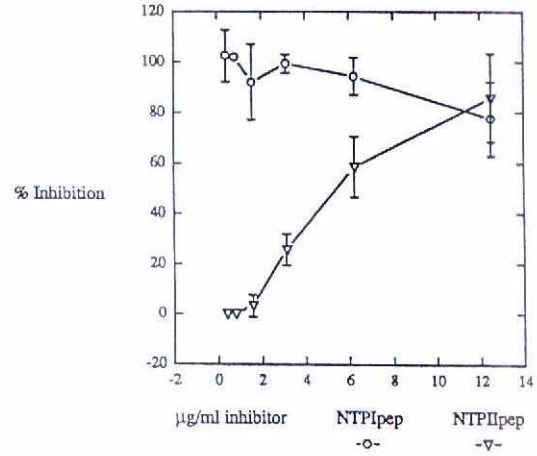

Figure 2. Competitive antigen ELISA. Plate coated with NTPIpep.

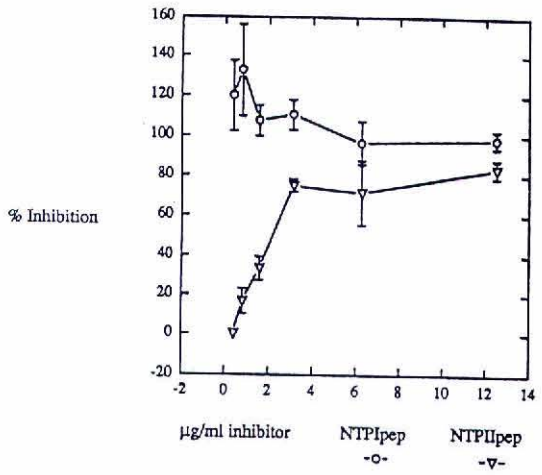

Figure 3. Competitive antigen ELISA. Plate coated with NTPIIpep.

concentrations and therefore much more efficiently than NTPIIpep. This would be expected since NTPIIpep has only 70% homology with the NTPIpep coating antigen. However it is interesting to observe that when NTPIIpep is used as the coated antigen (Fig. 3), NTPIpep, which has only 70% homology to the coated antigen, competes for antibody much more efficiently than NTPIIpep competes against itself. This is evident by the lower concentrations of NTPIpep needed for competition. These results clearly showed that NTPIpep is highly efficient at competing for antibody against itself and also against NTPIIpep, whereas NTPIIpep competes for antibody with much less efficiency for both coated antigens. The high antibody affinity to the NTPI peptide indicates that the NTPI peptide is the homologous epitope that has stimulated the antibody response, whilst the lower affinity NTPIIpep is the cross reactive heterologous epitope. Since only virulent strains contain the NTPI epitope, it would suggest that this patient was infected by a virulent strain of *T. gondii*. The use of this competitive antigen ELISA strategy needs to be assessed using sera from a range of virulent and avirulent experimental infections before such a strategy could be reliably used to detect infection by virulent strains of *T. gondii*. However, the results presented offer preliminary evidence that such technology is possible.

Indirect ELISA

Because the experiments used to identify this epitope used an anti IgA, IgM and IgG pooled conjugate, we have also used a standard indirect ELISA to determine the isotype of the antibody directed at this epitope. Using NTPIpep and NTPIIpep as coated antigens, serial dilutions of the primary serum and a 1/500 dilution of either anti-human IgG or anti-human IgM, it was found that IgG antibodies were detected, whereas IgM antibodies were not (data not shown). This establishes NTPIpep as a differential epitope which induces the formation of IgG antibodies.

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