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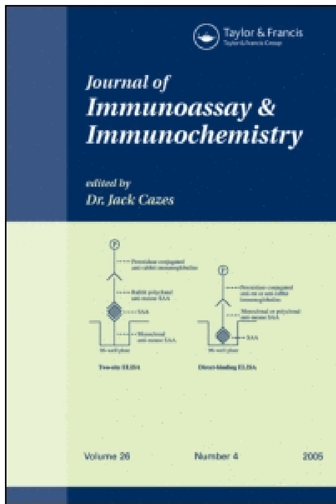

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HUMAN ANTIBODY RESPONSE TO THE NUCLEOSIDE TRIPHOSPHATE HYDROLASE OF *TOXOPLASMA GONDII*

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) that uses a recombinant protein of *Toxoplasma gondii* as antigen was used for the detection of specific antibodies in human sera. An antigenic portion of the *T. gondii* nucleoside triphosphate hydrolase (NTPase) was expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein with an apparent molecular mass of about 50000. A total of 118 *T. gondii* positive and 63 negative sera were examined. Seven % of the *T. gondii* positive sera, but none of the negative sera, reacted with the recombinant NTPase. Advantages and disadvantages that are associated with the use of fusion proteins as antigens in ELISA are discussed.

(KEY WORDS: *Toxoplasma gondii*, GST fusion protein, parasite-specific antibodies, pGEX vectors)

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INTRODUCTION

Toxoplasma gondii is a coccidian parasite of most warm-blooded animals including humans. Most *T. gondii* infections in humans are asymptomatic, but first exposure to the parasite during pregnancy may cause abortion or congenital malformations, and the disease is often fatal for immunosuppressed patients, such as those with acquired immunodeficiency syndrome (1). Therefore, several methods have been developed for the detection of *T. gondii* antibodies which involve a broad variety of

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serological tests using different kinds of antigens (2). Enzyme-linked immunosorbent assays (ELISA) are used increasingly, especially for epidemiological studies, as they can be easily and rapidly performed, objectively evaluated, and offer the possibility of automation. However, the usefulness of these tests in the diagnosis of toxoplasmosis has been limited by the inability to obtain standardized reagents, in particular antigens of constant quality, because _T. gondii_ is obligately intracellular. Hence antigens are always contaminated with the host cells in which this parasite is grown.

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The production of recombinant antigens in bacterial or yeast cells offers a potential for the development of highly standardized diagnostic tests with well-defined, reproducible, and inexpensive antigens. The endozoite stage of _T. gondii_ contains a nucleoside triphosphate hydrolase (NTPase) which constitutes about 8 % of the total cell proteins and circulates in the sera of mice from 3 to 56 days after intraperitoneal inoculation of _T. gondii_ cysts (3). In this study, we report the development of an ELISA that uses recombinant NTPase of _T. gondii_ for the detection of antibodies to it in human sera.

MATERIALS AND METHODS

Preparation of Antigens

T. gondii endozoites (TGE) of the RH strain were harvested from the peritoneal exudates of mice and disrupted by sonication as previously described (4). The gene fragment encoding an

antigenic portion of the T. gondii NTPase was subcloned from a lambda gt11 expression library into the plasmid pGEX-1N (5). Recombinant NTPase was expressed in E. coli strain JM105 as a glutathione S-transferase (GST) fusion protein and purified under non-denaturing conditions by adsorption to glutathione-agarose beads (6). Several preparations of recombinant NTPase with yields of 0.7-2.4 µg/ml of culture were combined to provide an antigen pool that was concentrated by ultrafiltration. GST was produced from cultures of E. coli containing pGEX-1N following the same procedure as for the recombinant NTPase. The protein concentration of the antigen preparations was determined by a Bradford dye-binding assay.

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Sera

The 118 T. gondii positive and 63 negative sera used here were obtained from 181 patients who were investigated at the Flinders Medical Centre between January and August 1989 for a variety of symptoms or conditions in which toxoplasmosis may be important. Discrimination of positive and negative sera was based on a routine diagnostic ELISA (4, 7). All sera were examined with TGE using the same serum dilutions and test reagents as for the recombinant NTPase antigen. Equal volumes of 12 sera that rendered optical density (O.D.) values $\geq$ 1.50 with TGE at a serum dilution of 1:10 were combined to provide a large amount of positive reference serum. Equal volumes of another 12 sera with O.D. values < 0.30 at a serum dilution of 1:10 were combined to provide a negative reference serum.

ELISA

Antigens were thawed and diluted to the working dilution with coating buffer (0.015 M carbonate 0.035 M bicarbonate, pH 9.6). Flat bottom polystyrene ELISA plates (Immuno Plate Maxisorp F96; A/S NUNC, Roskilde, Denmark) were coated with 100 µl of the antigen solution per well at 4 °C for at least 15 h and then washed five times with 0.01 M phosphate-buffered 0.15 M saline (PBS). The sensitized wells were post-coated with 100 µl of 5 % w/v BSA (bovine albumin; Commonwealth Serum Laboratories, Melbourne, Australia) in PBS at 37 °C for 1 h and then washed five times with PBS containing 0.05 % v/v Tween 20 (PBS-Tween). Serum samples were diluted with 1 % BSA in PBS-Tween and 100 µl of each sample was added to each respective well. The plates were incubated and washed as before. Commercially available goat anti-human IgG (H+L) conjugated to alkaline phosphatase (antibody concentration 0.6 mg/ml; Jackson Immunoresearch Laboratories, West Grove, PA., USA) was diluted to the working dilution with 1 % BSA in PBS-Tween and 100 µl was added to each well. The plates were incubated as before and then washed five times each with PBS-Tween and deionized water. Substrate solution was prepared by dissolving 5 mg p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets; Sigma Chemical Company, St. Louis, MO., USA) in 5 ml of 10 % w/w diethanolamine buffer with 0.5 mM magnesium chloride, pH 9.8; 100 µl was added to each well and the plates were incubated at 22 °C in the dark. The O.D. values were measured at a wavelength of 405 nm against a control well that had not received serum or conjugate.

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Determination of Optimum Conditions of the Assay

The optimal working dilution of each of the three antigens (TGE, recombinant NTPase, and GST) and of the conjugate was determined by chequerboard assays with serial dilutions of antigen and conjugate. For these assays, positive and negative reference sera were used at a dilution of 1:100 and the substrate reaction was stopped after 10 min by the addition of 25 µl of 2 M sodium hydroxide.

In order to determine the optimal serum dilution for the screening of human sera, the 24 sera that were used to construct the positive and negative reference sera were titrated from 1:10 to 1:1280. The O.D. was measured when the O.D. value for the positive reference serum at a serum dilution of 1:20 reached 1.50. For each single serum and for each serum dilution, the O.D. values obtained with the recombinant NTPase and with GST were used to calculate the difference in O.D. values between those two antigens (NTPase-GST). The serum dilution that rendered the highest difference in O.D. values measured for positive and negative sera and for recombinant NTPase and GST was chosen for the screening of all 63 negative and 118 positive sera.

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RESULTS

The chequerboard assay with TGE determined a working dilution of 1:200 (1 µg protein/well) for the antigen and of 1:1000 for the conjugate. By contrast, the chequerboard assay with recombinant NTPase determined a working dilution of 1:100 (2 µg protein/well) for the antigen and of 1:200 for the

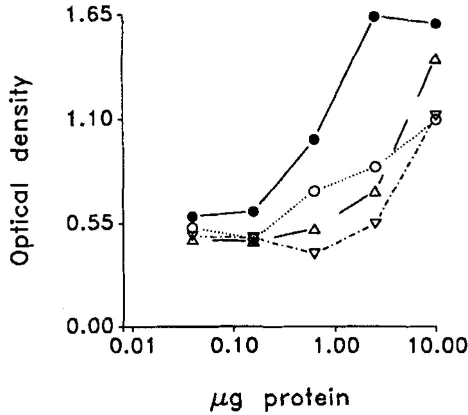

FIGURE 1: Titration of antigens in an ELISA (conjugate dilution 1:160). Data are shown for: recombinant NTPase with positive (●) and negative (○) reference serum; GST with positive (△) and negative (▽) reference serum.

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conjugate. GST was used at the same conditions as the recombinant NTPase. Figure 1 shows the O.D. values obtained with serial dilutions of recombinant NTPase and GST at a conjugate dilution of 1:160. A serum dilution of 1:80 was chosen for the screening of single sera as this dilution rendered the highest difference in O.D. values between the 24 positive and negative sera and between recombinant NTPase and GST (data not shown).

The examination of the 181 single T. gondii positive or negative sera at a serum dilution of 1:80 showed a broad distribution of O.D. values obtained with the recombinant NTPase and with GST over a wide O.D. range (Fig. 2). The level of antibodies varied among the single sera and resulted in a high standard deviation (SD) obtained for the assay with recombinant

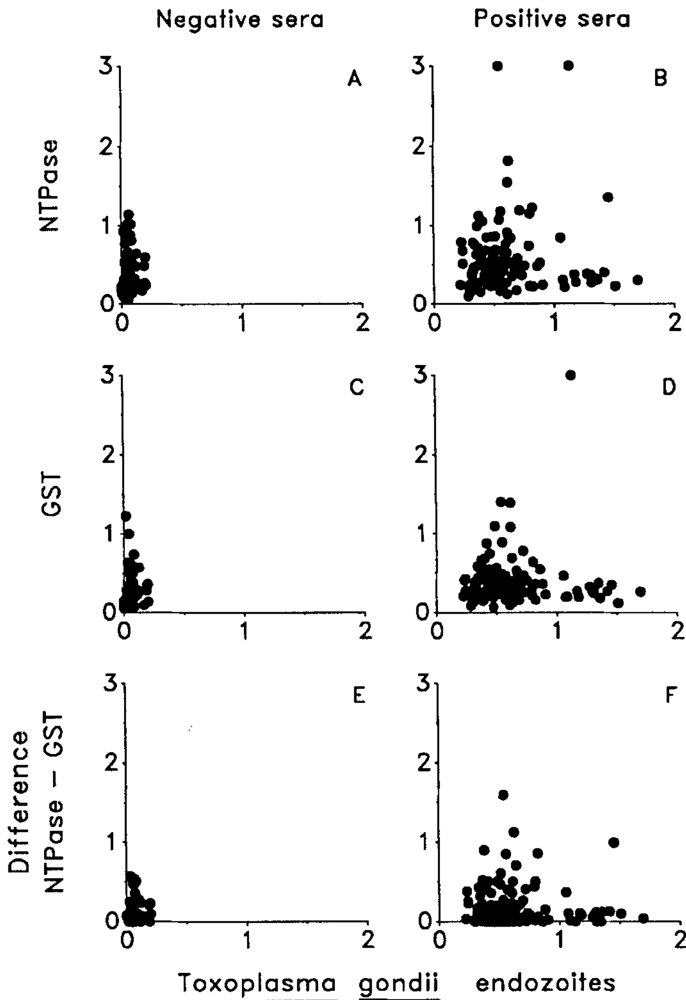

FIGURE 2: Y axes; optical densities obtained in ELISA using recombinant NTPase or GST as antigen, and the difference between them, for 63 negative sera (Panels A, C, and E, respectively), and the optical densities obtained in ELISA using recombinant NTPase or GST as antigen, and the difference between them, for 118 positive sera (Panels B, D, and F, respectively): X axes; optical densities obtained in ELISA using *T. gondii* endozoites as antigen (all Panels).

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TABLE 1

O.D. Values for 118 *T. gondii* Positive and 63 Negative Sera at a Serum Dilution of 1:80

Antigen	O.D.			
	Positive sera		Negative sera	
	Range	$\bar{x} \pm$ SD	Range	$\bar{x} \pm$ SD
TGE	0.22 - 1.70	0.61 ± 0.31	0.00 - 0.20	0.06 ± 0.05
NTPase	0.10 - 3.00	0.57 ± 0.45	0.06 - 1.14	0.43 ± 0.29
NTPase-GST	0.00 - 1.60	0.18 ± 0.26	0.00 - 0.57	0.12 ± 0.15

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NTPase (Table 1). Seven % of the *T. gondii* positive sera showed O.D. values ≥ the mean plus three SD (0.57) of the negative control group.

DISCUSSION

ELISA for the diagnosis of several infections caused by organisms that are difficult to obtain pure in quantity (8-12) have recently been improved by the use of recombinant antigens. However, we are unaware of previous studies reporting the use of recombinant *T. gondii* antigens in ELISA.

We used the plasmid pGEX-1N for the expression of an antigenic portion of the NTPase of *T. gondii*. This gene fragment was subcloned into pGEX-1N and the NTPase was expressed in *Escherichia coli* as a GST fusion protein with an apparent molecular mass of about 50000. The recombinant NTPase was

soluble in aqueous solutions which enabled its use as an ELISA antigen.

A problem with the use of GST fusion proteins as antigens in an ELISA for the examination of human sera may be the high prevalence of antibodies directed to GST in these sera. Specific antibodies to GST have been shown to occur in the majority of human sera (13). We therefore included GST expressed by pGEX-1N in E. coli as a control antigen in this study and subtracted the O.D. values that were obtained with this antigen from the O.D. values obtained with the recombinant NTPase. Using these values, 7 % of the T. gondii positive sera, but none of the negative sera, reacted with the recombinant NTPase.

The high level of antibodies to GST in some human sera (13) may mask a low level of antibodies directed to the NTPase part of the fusion protein used in this study. Cleavage of the GST carrier from the fusion proteins (6) may improve the sensitivity of assays using recombinant antigens that originate from pGEX vectors, especially if an unfavourable ratio between the GST part and the part of the desired antigen (1:2 in this study) exists.

The quality of ELISA depends on the specificity, affinity, and avidity of the antigens and antibodies involved. Hedman et al. (14) have recently found that IgG antibodies produced early in toxoplasmosis have lower avidity for TGE than those IgG antibodies produced later in the infection. We believed that as NTPase is antigenic and constitutes up to 8 % of the total endozoite protein, it would be a good candidate to test for recognition by sera from naturally infected humans. However, the

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results of the assay used here, are consistent with the hypothesis that the affinity of the recombinant NTPase or the avidity of antibodies directed to epitopes presented by the recombinant NTPase may not be high.

It is likely that this ELISA for the detection of antibodies to T. gondii can be improved by the inclusion of additional recombinant antigens with different qualities. This has already been shown for P. falciparum where a mixture of three recombinant polypeptides significantly increased the sensitivity, even when each single polypeptide was present at a lower concentration in the assay based on this mixture than in the assays based on a single recombinant polypeptide (12). Work to test this hypothesis by the inclusion of other recombinant T. gondii antigens is at present underway in our laboratory.

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