Clin. exp. Immunol. (1985) **62,** 262–269.

Serodiagnosis of toxoplasma infection using a purified parasite protein (P30)

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(Accepted for publication 26 April 1985)

SUMMARY

The major surface protein (P30) of *Toxoplasma gondii* has been purified by immunoabsorption with anti-P30 monoclonal antibodies linked to a glutardialdehyde activated affinity absorbant. SDS-PAGE analysis of the eluted material followed by silver staining showed only a single band of 30,000 mol wt. Western blotting using antibodies from a rabbit immunized with purified P30 against the total *Toxoplasma* extract separated by SDS-PAGE again revealed an unique antigen of 30,000 daltons. The presence of repeated epitopes within P30 was confirmed by a two-site/one-antibody radiometric assay with the purified protein. Sandwich ELISA procedures with purified P30 clearly demonstrated that all 37 tested patients with acute toxoplasmosis presented significantly high levels of IgM anti-P30 antibodies. In addition, all 40 tested patients with chronic toxoplasma infection also showed high IgG anti-P30 antibody levels. These findings represent an essential step for the development of new reagents for the diagnosis of toxoplasmosis.

Keywords toxoplasmosis IgM serodiagnosis IgG serodiagnosis purified protein

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that is probably capable of infecting all species of mammals, including man (Siim, Biering-Sorensen & Moller, 1963). Characterization and isolation of Toxoplasma antigens would be of great interest for the development of newer diagnostic methods and for the study of the immune response to the parasite. For this reason, several laboratories have produced monoclonal antibodies (mAb) against the surface of the tachyzoites (Handman, Goding & Remington, 1980; Sethi, Endo & Brandis, 1980; Johnson *et al.*, 1981; Kasper, Crabb & Pfefferkorn, 1983; Rodriguez *et al.*, 1985). Most of these antibodies recognized a major radioiodinated surface protein with an apparent mol wt of 27,000 to 30,000, as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which has been named P30 (Kasper *et al.*, 1983; Rodriguez *et al.*, 1985). P30 contains a single immunodominant region with two or more identical epitopes as determined by a two-site/one antibody radiometric assay with mAb (Rodriguez *et al.*, 1985). Passive transfer experiments (Johnson, McDonald & Neoh, 1983) and active immunization with partially purified antigens (Araujo & Remington, 1984) suggested a role for P30 as a protective immunogen against toxoplasmosis.

The presence of antibodies against P30 in the serum of patients with either recent or past Toxoplasma infection has been demonstrated by different techniques (Handman *et al.*, 1980; Kasper *et al.*, 1983; Sharma *et al.*, 1983). Recently, P30 has been purified by immunoadsorption

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with mAb (Kasper *et al.*, 1983). In the present study we also purified P30 and demonstrated that all patients with both acute (IgM) and chronic (IgG) toxoplasmosis have significant levels of anti-P30 antibodies.

MATERIALS AND METHODS

Toxoplasma gondii antigen. The virulent RH strain of *T. gondii* was maintained by serial intraperitoneal passages in mice. The parasites were harvested from the peritoneal cavity of mice infected 3 days earlier. They were passed through a nylon wool column to remove cell debris and washed three times with phosphate-buffered saline (PBS), pH 7·2 at 1,200 ***g*** for 20 min. The final pellet was dissolved in 0·5% Nonidet P40 (Sigma chemical Co, St Louis, Missouri, USA) in PBS containing 100 u/ml of the protease inhibitor aprotinin (Sigma). After 1 h incubation at 4°C the extract was centrifuged at 10,000 ***g*** for 20 min at 4°C. The supernatant was used as the source of antigen and is referred as the Toxoplasma extract.

Monoclonal antibodies. Hybridomas secreting mAb against P30 have been described elsewhere (Rodriguez *et al.*, 1985). Ascitic fluids were produced in pristane-primed Balb/C mice and the mAb were purified by a 50% ammonium sulphate precipitation followed by a chromatography on DEAE-Trisacryl (IBF, Villeneuve la Garenne, France). Enzyme labelling of purified anti-P30 mAb was performed as described by Engvall & Perlman (1971). The only modification was the use of β-galactosidase (Boehringer, Mannheim, FRG) instead of alkaline phosphatase.

Human sera. The sera from the following groups of subjects were used: (a) 47 patients who were acutely infected with *T. gondii* and in whose sera the presence of IgM toxoplasma antibodies had been confirmed by indirect immunofluorescence and/or by haemagglutination before and after treatment with β-mercaptoethanol; (b) 40 patients who were positive for IgG anti-*T. gondii* antibodies but negative for IgM antibodies; (c) 25 patients with other protozoan parasitic diseases (Leishmaniasis, Chagas' disease); (d) 24 patients with seropositive rheumatoid arthritis; (e) 35 individuals who had no serological evidence of parasitic diseases nor immunopathological disorder.

Immunoadsorption of T. gondii *antigens.* A pool of purified mAb anti-P30 (Rodriguez *et al.*, 1985) was coupled to glutardialdehyde activated affinity absorbent (Boehringer, Mannheim, West Germany) according to the manufacturer's recommended method. Briefly, 1 g of affinity adsorbent was suspended in 5 ml of 0·9% NaCl containing 20 mg/ml of mAb. After shaking for 4 h at room temperature, the carrier was transferred to a chromatographic column and washed with 1·5% NaCl until the eluate was free of protein (OD < 0·005). Remaining free aldehyde groups on the carrier were saturated with 0·3 M ethanolamine-HCl, pH 7·5 for 1 h. After washing sequentially with 50 ml 0·9% NaCl, 50 ml 0·5 M glycine, pH 3·75, and 100 ml 0·9% NaCl, the immunoadsorbent was stored at 4°C.

Isolation of P30 was carried out overnight at 4°C by pumping in a continuous closed system 10 ml of Toxoplasma extract (200 mg) with a flow rate of 2 ml/h through the immunoadsorbant column. The chromatographic system was then opened and washed with 0·9% NaCl until an effluent OD < 0·005 was obtained. Afterwards the bound antigen was eluted with 0·1 M triethylamine pH 11·5 and rapidly neutralized with 1 M HCl until the pH was 7·2. After exhaustive dialysis against PBS, pH 7·2, the eluted proteins were stored at −70°C.

Polyacrylamide gel electrophoresis. Antigen preparations were dissolved in sample buffer and boiled for 3 min as previously described (Dissous, Grych & Capron, 1982). Electrophoresis was carried out in 0·1% SDS discontinuous 13% polyacrylamide gels according to the method of Laemmli (1970). The gels were then either subjected to Western blotting or were stained. For silver staining (Bio-Rad Laboratories, Muenchen, FRG), we followed the manufacturer's recommended method.

Western blot analysis. Proteins were transferred from the running gel to a nitrocellulose membrane (Schleicher & Schüll, Dassel, FRG) as described by Towbin, Staehelin & Gordon (1979). The transfer was performed for 3 h at 60 V/0·3 A (Bio-Rad Trans Blot apparatus). The electrophoretic blots were then washed four times for 15 min each in PBS-0·3% Tween 20 with constant agitation. The cut paper strips were then incubated with either normal or immunized

rabbit sera diluted 1/2000 in PBS-Tween for 1 h. After three washes as above the fixed antibodies were revealed by incubation with horseradish peroxidase-conjugated goat anti-rabbit-IgG antibodies (Miles-Yeda, Ltd., Israel) at a dilution of 1/2000 in PBS-Tween for 1 h at room temperature with constant agitation. The blots were once again washed three times in PBS-Tween and were soaked in a solution of 0·5 mg/ml of diaminobenzidine 0·1% H_2O_2, PBS pH 7·2. The colour development was stopped by washing with distilled water. These blots were photographed immediately.

Immunoradiometric assay. This was performed as previously described (Zavala *et al.*, 1983; Rodriguez *et al.*, 1985). Briefly wells of a microtitre plate were coated overnight at 4°C with 50 μl of a 10 μl/ml solution of purified anti-P30 mAb. After three washes, PBS-3% BSA was left in the wells for 1 h at room temperature. 35 μl of different concentrations of antigen was added to separate wells and the plate was incubated at room temperature for 4 h. After three washes with PBS-BSA containing 0·5% Nonidet-P40, 30 μl (1–10 ng, 1×10^5 ct/min) of ^{125}I-labelled mAb (the same used to coat the wells) was added to each well and the plate left for 1 h at room temperature. The wells were then washed three times, dried and counted.

Sandwich ELISA. Wells of a microtitre plate were coated overnight at 4°C with 200 μl of either a 5 μg/ml solution of purified P30 antigen or Toxoplasma extract in PBS, pH 7·2. After three washes in saline containing 0·4% Tween, the wells were kept for 2 h at room temperature with PBS containing 0·1% BSA and 0·1% Tween. After three washes in saline-Tween, 200 μl of human sera diluted 1/400 for ELISA-IgG and 1/6,400 for ELISA-IgM in PBS-0·1% Tween were added to the wells and kept for 3 h at room temperature. The wells were once again washed as above and incubated for 1 h at room temperature with 200 μl of β-galactosidase-conjugated goat anti-human IgG (ELISA-IgG) or anti-human IgM (ELISA-IgM) antibodies (Zymed Laboratories Inc., San Francisco, California, USA) diluted 1/500 in PBS-Tween. After three washes in Saline-Tween, the antibody was detected by adding 200 μl of an indicator solution containing 0·014 M 2-Nitrophenil-β-D-galactopyranoside, 0·003 M $MgCl_2$ and 0·1 M β-Mercaptoethanol. The colour development was stopped by adding 100 μl of 1 M Na_2CO_3 and absorbance was measured at 405 nm using a Titertek Multiskan (Flow Laboratories, McLean, Virginia, USA). A serum was considered positive when its absorbance was at least twice that of the mean of the negative control sera.

IgM capture ELISA. A modification of the method described by Naot & Remington (1980) was used. Wells of a microtitre plate were coated overnight at 4°C with 250 μl of affinity purified IgG fraction of a sheep anti-human IgM (μ specific) (Institut Pasteur, Lille, France) diluted 10 μg/ml in PBS, pH 7·2. After three washes in Saline-0·4% Tween, the plates were incubated for 2 h at room temperature with PBS containing 0·1% gelatine and 0·5% Tween. After three washes in saline-Tween, 200 μl of human sera diluted 1/100 in PBS-gelatine-Tween were added to the wells and kept for 2 h at 37°C. After three washes as above, 200 μl of either a 50 μg/ml solution of purified P30 or 200 μg/ml of Toxoplasma extract in PBS-gelatine-Tween were added to the wells and incubated overnight at 4°C. The wells were washed three times with saline-Tween and incubated for 3 h at room temperature with 200 μl of β-galactosidase-conjugated anti-P30 mAb prepared as described above. The enzymatic reaction and measurement was performed as in the sandwich ELISA.

RESULTS

Purification of protein P30

The use of immunosorbent with mAb covalently attached to cyanogen bromide-activated Sepharose to purify P30 has been described earlier by Kasper *et al.* (1983). Initially, we have tried this same procedure with our mAb to isolate P30. Nevertheless, the recovery of purified P30 that we obtained (1–2% of the total protein) was relatively weak and not sufficient to the purpose of this study. For this reason, we used another affinity absorbent, activated by glutardialdehyde, and to increase the yield of recovered P30, a continuous chromatographic system in which the Toxoplasma extract passed several times through the immunosorbent column, was also used. In these conditions, about 5% of the total protein was recovered as purified P30. The immunosorbent column was regenerated and used twice more in this study. A total of 15 mg of protein was obtained.

Characterization of purified P30

First, our preparation of protein P30 was analyzed by SDS-PAGE followed by the highly sensitive silver strain, which is able to detect minor contaminating proteins in acrylamide gels. Figure 1 shows a comparison of the silver-stained Toxoplasma extract (40 μg-track A) with 10 μg of our preparation of P30 (track B). The silver stain of the protein purified by immunosorption showed only a single band of 30,000 daltons. Minor contaminating proteins were only observed when 20 μg of our purified preparation was analysed by silver stain. This data is not shown because the gel was overloaded with the 30,000 mol wt protein.

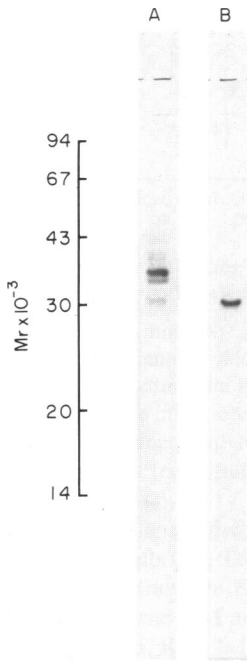

Fig. 1. A comparison of silver stain of 40 μg of total Toxoplasma extract (A) and 10 μg of purified protein P30 (B) after resolution by polyacrylamide gel electrophoresis in the presence of SDS.

In order to investigate the immunogenicity of our preparation of P30, 150 μg of the protein diluted in Freund's complete adjuvant was intradermally injected into a rabbit and the serum obtained was tested against the Toxoplasma extract by Western blotting. A rabbit antiserum prepared against the total Toxoplasma extract was used in the same experiment as control. Figure 2 shows that the serum of the rabbit immunized with protein P30 only recognized a single antigen of 30,000 daltons (track C). The antiserum produced against the Toxoplasma extract reacted with more than 15 antigens of different mol wt (track B). All these findings indicate that the protein P30 obtained is chemically and antigenically purified.

Demonstration that purified P30 contains two or more identical epitopes

In previous work, Rodriguez *et al.* (1985) have shown that P30 present in the Toxoplasma extract contains repetitive epitopes. In the present study we used the same methodology, a two-site/one-antibody immunoradiometric assay, to compare the multivalency of expression of a single epitope within both the purified molecules of P30 and in the Toxoplasma extract containing P30. In this assay the same mAb anti-P30 is used both in solid phase and as the developing reagent. To avoid aggregation of P30 molecules the incubation of the protein with the solid phase-bound mAb and the following washes were performed in the presence of Nonidet P40, a non-ionic detergent. Figure 3 shows that the expression of two or more identical epitopes within P30 molecules is at least four times greater in the purified protein than in the Toxoplasma extract.

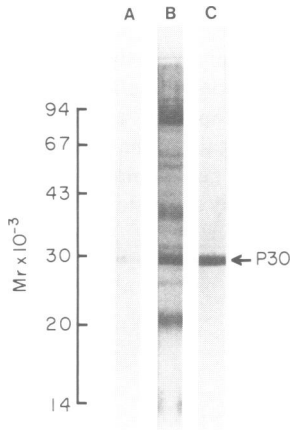

Fig. 2. Reactivity of rabbit antibodies to Toxoplasma extract after electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose membranes. (A), antigens recognized by normal rabbit sera. (B), antigens recognized by IgG antibodies of the rabbit immunized with the total Toxoplasma extract. (C), antigens recognized by IgG antibodies of the rabbit immunized with purified protein P30.

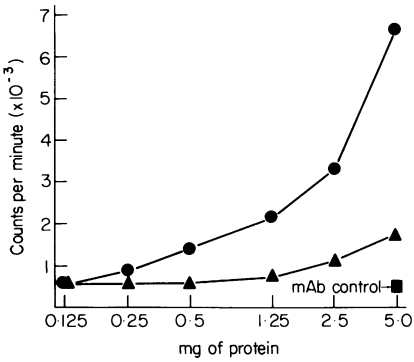

Fig. 3. Immunoradiometric assay for detection of repetitive epitopes within P30. Toxoplasma extract (▲) or purified P30 (●) were incubated in wells of microtitre plates coated with anti-P30 mAb. After washing, the amount of antigen bound to the solid phase was measured by adding to the wells an excess of the same mAb radiolabelled with ^{125}I.

Serodiagnosis of chronic (latent) toxoplasmosis using purified P30

In this study we used a sandwich ELISA, in which the wells of a microtitre plate were coated with purified P30. After contact with the test sera diluted 1/400, the IgG antibodies bound to P30 were revealed with a β-galactosidase-conjugated goat anti-human IgG. Initially, we did some preliminary experiments in order to determine the concentrations of the reagents used in the different steps of the test. As we had limitations concerning the amount of our main reagent, purified protein P30, a complete investigation of all the parameters involved in this kind of method could not be performed. Finally, we decided to use the sandwich ELISA described in Materials and methods, which may not be ideal for serodiagnosis, but gave significant results on the detection of antibodies in sera of patients with toxoplasmosis (Fig. 4). In fact, all 40 subjects with chronic toxoplasmosis and 29 out of 47 patients with an acute infection showed significant levels of anti-P30

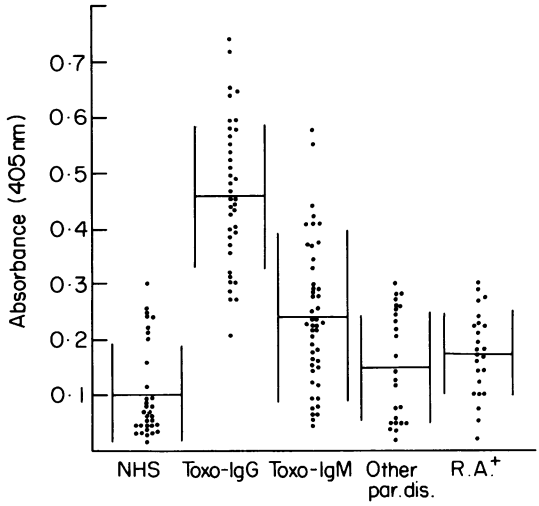

Fig. 4. Sandwich ELISA for detection of anti-P30 IgG antibodies. NHS: Normal Human Serum; Toxo-IgG: patients with chronic (latent) toxoplasmosis; Toxo-IgM: patients with acute acquired toxoplasmosis; Other par. dis.: Patients with other parasite disease (Leishmaniasis, Chagas' disease); R.A.$^+$: patients with seropositive rheumatoid arthritis.

IgG antibodies. A few patients with other parasitic infections (Leishmaniasis, Chagas' disease) and with rheumatoid arthritis gave a weak positive reaction. Nevertheless, the mean levels of these groups of patients were not significantly different from that of the control group.

Serodiagnosis of acute acquired toxoplasmosis using purified P30

The detection of anti-P30 IgM antibodies in patients with Toxoplasma infection was carried out by two different methods. First, we used the sandwich ELISA described above with minor modifications: the sera were diluted 1/6,400 and the IgM antibodies bound to P30 were revealed with an enzyme-conjugated goat anti-human IgM. Results in Fig. 5 indicate that most of patients with serologic characteristics of recent or acute Toxoplasma infection present significant levels of anti-P30 IgM antibodies. However, some of the subjects from the other groups studied also showed a positive test. False-positive results for IgM were previously described with this kind of technique using a total *T. gondii* antigen extract (Camargo *et al.*, 1978; Noat & Remington, 1980). In view of these findings and in an attempt to gain better specificity, we used another procedure, the IgM

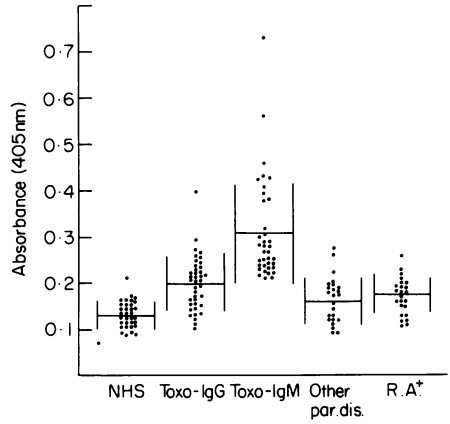

Fig. 5. Sandwich ELISA for detection of anti-P30 IgM antibodies. Patient groups as in Fig. 4.

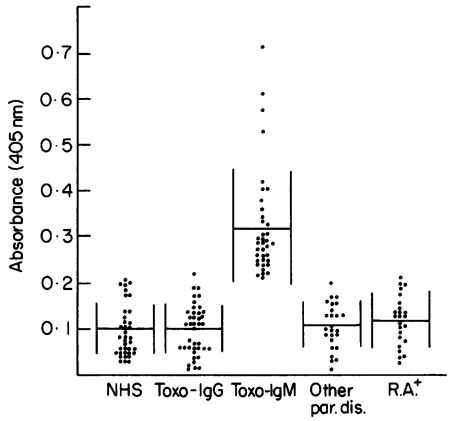

Fig. 6. IgM capture ELISA for detection of anti-P30 IgM antibodies. Patient groups as in Fig. 4.

capture ELISA with purified P30. In this method, the IgM of the test sera are first attached by a sheep IgG anti-human IgM previously coated to the wells. In a second step the purified P30 is added, and the protein specifically bound to IgM, is revealed by the labelled anti-P30 mAb. Using this method, specific IgM anti-P30 antibodies were detected in serum of all patients with acute toxoplasmosis (Fig. 6).

DISCUSSION

Previous analytical studies have shown the presence of anti-P30 antibodies in sera of patients with Toxoplasma infection (Handman *et al.*, 1980; Sharma *et al.*, 1983). In addition, in a preliminary investigation purified P30 has been used in an ELISA test to demonstrate the presence of specific antibodies in the serum of three patients with convalescent toxoplasmosis (Kasper *et al.*, 1983). More recently, we partially inhibited the binding of specific human antibodies to a Toxoplasma antigen extract by adding anti-P30 mAb (Rodriguez *et al.*, 1985). The present investigation has demonstrated that purified P30 can be used as a single antigen for serodiagnosis of both acute and chronic toxoplasmosis.

Purification of protein P30 by immunosorption with mAb has been previously described (Kasper *et al.*, 1983). In the present work, by using a similar procedure, we easily obtained significant amounts of this protein. Several technical approaches have demonstrated the purity of P30. The silver stain of the protein purified by immunosorption in SDS-PAGE showed only a single band of 30,000 daltons (Fig. 1). Moreover, when the IgG antibodies of the rabbit immunized with the purified protein were tested against the total Toxoplasma extract by western blotting, only a single antigen of 30,000 mol wt was revealed.

Recently, we have demonstrated that protein P30 present in Toxoplasma extract contains a single immunodominant region composed of two or more identical epitopes (Rodriguez *et al.*, 1985). This immunochemical characteristic was noticed on a weight basis to be at least four times greater with purified P30. Repeated epitopes in major surface antigens of protozoa has been described earlier with malaria parasites (Godson *et al.*, 1983; Zavala *et al.*, 1983).

The major observation of our work is that all patients with a classical positive serology of toxoplasmosis present in their sera significant levels of anti-P30 antibodies. The sandwich ELISA procedures used throughout this investigation have been previously described (Voller *et al.*, 1976; Camargo *et al.*, 1978; Naot & Remington, 1980). The major modification was in the expression of the results. Usually, the end-point serum dilution that exhibits twice the reactivity of the mean absorbance of the negative control sera gives better results than the absorbance values of a single serum dilution (Naot & Remington, 1980). Unfortunately, due to the limitations of our main reagent, purified P30, an exact titration of each studied serum could not be done. This same reason prevented us from performing a complete investigation of all parameters involved in this kind of method which certainly would increase its sensitivity.

IgG antibodies against purified P30 were detected in all patients with a chronic toxoplasmosis and in some patients with an acute infection. Most of the patients from the other studied groups with a weak significant level of IgG anti-P30 antibodies also presented a weak positive serology (data not shown). As the sensitivity of the sandwich ELISA could not be compared with the classical indirect immunofluorescence and haemagglutination techniques, it is possible that those few patients without a positive toxoplasmosis serology and showing a weak significant level of anti-P30 antibodies could have a previously undetected chronic infection.

All patients with serologic characteristics of recent or acute toxoplasmosis showed significant levels of IgM anti-P30 antibodies by two different ELISA methods. A few false-positive results were observed with the sandwich ELISA. The same problem of specificity has been previously noticed with this method (Naot & Remington, 1980). However, with the IgM capture ELISA, this problem was solved and only the patients with an acute infection presented significant IgM anti-P30 antibody levels.

In conclusion, the fact that all patients with both acute and chronic toxoplasmosis presented significant high levels of anti-P30 antibodies represents an essential advance for the development of new reagents for the diagnosis of this parasitic disease.

The authors wish to thank Marie-Pierre Fourmaux, Anita Caron, Thérèse Lepresle and Hubert Caron for technical assistance and Marie-France Massard and Claudine Colson for secretarial help. This work was supported by grants from ANVAR (X 8408084N).

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