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# Expression, Characterization, and Serologic Reactivity of Recombinant Surface Antigen P22 of *Toxoplasma gondii*

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**The utility of recombinant *Toxoplasma gondii* surface antigen P22 for the detection of specific *T. gondii* antibodies in human sera was evaluated. Polymerase chain reaction was used to produce a 438-bp fragment of the P22 gene; the fragment corresponded to the amino acids predicted to be in the processed, native antigen. The fragment was subcloned into pGEX-2T and was expressed in *Escherichia coli* as a glutathione-*S*-transferase (GST) fusion protein. The fusion protein was purified in a soluble form and was found to be recognized by sera from infected individuals in immunoblots and an enzyme-linked immunosorbent assay. Immunoglobulin G antibodies in sera from 31 acutely infected patients in general reacted more strongly to the fusion protein than did those in sera from 31 patients with the chronic infection. None of the sera from a panel of 26 seronegative controls reacted with the fusion protein in immunoblots or an enzyme-linked immunosorbent assay. When the recombinant P22 portion of the fusion protein was separated from the GST partner by cleavage with thrombin, it retained its immunoreactivity and its electrophoretic mobility in polyacrylamide gels was found to be similar to that of native P22. By a modification of the published method for purification of the foreign polypeptide from the GST carrier, the recombinant P22 was readily purified to homogeneity by thrombin cleavage of the fusion protein while it was adsorbed to glutathione agarose.**

Diagnosis of infection with *Toxoplasma gondii* in humans is of considerable importance, especially in pregnant women and immunosuppressed patients. *T. gondii* infection acquired during pregnancy may result in permanent damage to the fetus. Toxoplasmosis in patients who are severely immunocompromised (e.g., patients with AIDS) frequently causes a life-threatening encephalitis. At present, the production of serologic tests for the diagnosis of infection with *T. gondii* by antibody detection requires isolation of the whole organism from the peritoneal fluid of acutely infected mice or from tissue culture. The commercial production of reliable serologic tests is often limited by difficulties in standardizing reagents, including toxoplasma antigen preparations. By using cloned antigens, large quantities of purified reagents can be prepared in bacterial expression systems. These antigens can be isolated relatively inexpensively and offer the potential for standardization of serologic tests that use *T. gondii* antigens. Only very recently has this strategy been attempted for study of its potential for the diagnosis of *T. gondii* infection. Johnson and colleagues (8, 18, 19) have cloned several antigens that are recognized by sera from infected humans and have tested purified preparations of these recombinant fusion proteins in an enzyme-linked immunosorbent assay (ELISA) with a panel of sera from individuals infected with *T. gondii*. They identified two recombinant antigens that were recognized by 68% of the sera from a panel of individuals with acute toxoplasmosis, while only 14% of the sera from a panel of patients with the chronic infection recognized the same antigens.

The use of other recombinant antigens of *T. gondii* in serologic tests such as ELISA should help improve our ability to discriminate between acute and chronic infections (17). In a recent series of studies from our laboratory, we cloned and sequenced the surface protein P22 of *T. gondii* and found in preliminary studies that the β-galactoside fusion protein reacts with antibodies in the sera of infected humans (11). Because of the difficulties encountered in purification of the β-galactoside fusion protein, we attempted to subclone the P22 gene into another bacterial expression system in order to produce a soluble, purified recombinant P22 that would be applicable for use in serologic tests and that could be provided in large amounts for commercial use.

## MATERIALS AND METHODS

**Enzymes, reagents, and chemicals.** Restriction enzymes, T4 DNA ligase, and pGEX-2T vector DNA were purchased from Pharmacia (Piscataway, N.J.). Vent DNA polymerase and reaction buffers were from New England Biolabs (Beverly, Mass.). Bacterial alkaline phosphatase was from Bethesda Research Laboratories (Gaithersburg, Md.). Thrombin, glutathione agarose, and reduced glutathione were from Sigma Chemical Co. (St. Louis, Mo.). Horseradish peroxidase (HRPO)-conjugated goat anti-mouse immunoglobulin G (IgG) and HRPO-conjugated goat anti-human IgA, IgG, and IgM were from Caltag Laboratories (San Francisco, Calif.).

**PCR.** The oligonucleotides 5′-GGGGGATCCACCACC GAGACGCCAGC-3′ and 5′-GGGGAATTCTTGCCCGTG AGAGACACAG-3′, designated primers 1 and 2, respectively, were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.). The polymerase chain reaction (PCR) was carried out in 100 μl containing 1 μM oligonucleotide primers–200 μM (each) deoxynucleoside triphosphates in Vent buffer (New England Biolabs) supple-

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mented with 2 mM $MgSO_4$. The template DNA was 100 pg of a 919-bp *Eco*RI fragment from the full-length P22 cDNA clone c88 (11). The reaction was incubated at 94°C for 5 min prior to the addition of 5 U of Vent DNA polymerase and 100 μl of light mineral oil. Amplification was carried out at 94°C for 1 min, 50°C for 2 min and 30 s, and 72°C for 2 min and 30 s for a total of 30 cycles in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, Mass.) by following recommended guidelines (14). Reactions were analyzed by agarose gel electrophoresis to verify the size and uniformity of the PCR products. To facilitate direct subcloning of the fragments, primers 1 and 2 were synthesized with *Bam*HI and *Eco*RI sequences at their 5′ ends, respectively. After phenol extraction, chloroform extraction, and ethanol precipitation, 1 μg of the PCR product was digested with *Eco*RI and *Bam*HI for 3 h and electrophoresed on an agarose gel (PurElute; Invitrogen, San Diego, Calif.). The insert was isolated by electrophoresis onto a DEAE-cellulose membrane (3) and was designated v22.

**Subcloning of two P22 inserts into pGEX vectors.** The first insert subcloned into pGEX-2T was the full-length P22 cDNA insert from λgt11 recombinant phage c88. Phage DNA was digested with *Eco*RI endonuclease, and the insert was purified by agarose gel electrophoresis as described above. After the addition of a 24-bp *Bam*HI-*Eco*RI adapter (Boehringer Mannheim, Indianapolis, Ind.), the insert was ligated into vector pGEX-2T that had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The adapter was used to maintain the correct reading frame, and the resulting recombinant clone was designated pGEX-2T-c88. The second insert subcloned into pGEX-2T was the v22 insert that was synthesized by PCR and purified as described above. The v22 insert was constructed with a *Bam*HI site at the beginning of the open reading frame and an *Eco*RI site at the end, allowing the insert to be inserted directionally into pGEX-2T vector that had been digested with *Bam*HI and *Eco*RI. The resulting recombinant clone was designated pGEX-2T-v22. The v22 insert from this clone was subcloned into M13mp18 and was sequenced by using the Sequenase kit (United States Biochemicals, Cleveland, Ohio).

**Production of fusion proteins in *Escherichia coli*.** For expression of fusion proteins, competent *E. coli* JM101 (Stratagene, La Jolla, Calif.) was transformed with parental or recombinant pGEX-2T DNA. Fusion proteins or glutathione-*S*-transferase (GST) wild-type protein was prepared from bacterial cultures of pGEX-2T-c88, pGEX-2T-v22, or pGEX-2T as described previously (15). Briefly, overnight cultures were diluted 1:10 in 1 liter of fresh medium and grown for 1 h at 37°C and were then induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.4 mM. After a further 3 h of growth, cells were pelleted and resuspended in 10 ml of phosphate-buffered saline (PBS; 10 mM $Na_2HPO_4$ [pH 7.2], 150 mM NaCl). Cells were lysed on ice by multiple rounds of sonication and Triton X-100 (Sigma) was added to 1%. The lysed cells were centrifuged at 10,000 × *g* for 5 min at 4°C, and the resulting supernatant was mixed with 4 ml of a 50% slurry of preswollen glutathione-agarose beads and agitated at room temperature for 30 min. The adsorbed beads were collected by centrifugation at 500 × *g* and washed three times with 50 ml of PBS containing 1% Triton X-100 and two times with PBS containing 0.1% Triton X-100, and the fusion protein was eluted by resuspending the beads two times in 4 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM free reduced glutathione.

**Thrombin cleavage.** The recombinant P22 (rP22) portion of the v22 fusion protein was separated from the GST fusion partner by cleavage with thrombin at the site encoded immediately upstream of the cloning site of the pGEX-2T vector as described previously (15). About 100 μg of purified fusion protein was diluted in thrombin cleavage buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2.5 mM $CaCl_2$), 1 U of thrombin was added (Sigma), and the solution was incubated at room temperature for 90 min. A portion of the reaction was analyzed by polyacrylamide gel electrophoresis (PAGE) (9) to determine the extent of cleavage.

In order to isolate rP22 and eliminate the GST fusion partner, the fusion protein was digested with thrombin while it was still bound to glutathione agarose beads. The v22 fusion protein was purified as described above, except that the fusion protein was not eluted from glutathione agarose with free glutathione. Instead, after the PBS washes, an additional 50-ml wash was performed with thrombin cleavage buffer. The adsorbed beads (2-ml bed volume) containing about 12 mg of bound fusion protein were suspended in 4 ml of thrombin cleavage buffer and were incubated with 100 U of thrombin at room temperature for 90 min, with agitation. To determine the extent of cleavage, 200 μl of the slurry was removed and analyzed by PAGE. The remainder of the slurry was centrifuged at 500 × *g*, and the rP22 protein was recovered in the supernatant. This procedure yielded rP22 with no detectable contamination with the v22 fusion protein or the GST partner on polyacrylamide gels stained with Coomassie brilliant blue.

***T. gondii* lysates.** *T. gondii* tachyzoites of the RH strain were harvested from the peritoneal cavity of Swiss-Webster mice as described previously (12). Reduced lysate was prepared by resuspension of tachyzoites in buffer containing 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, plus protease inhibitors and treatment as described previously (7). Nonreduced lysate was prepared by resuspension of tachyzoites in PBS containing 0.5% Nonidet P-40 and incubation at room temperature for 30 min.

**PAGE and immunoblot analysis.** Proteins were analyzed by SDS-PAGE in 10 to 15% gradient slab gels (9). The reactivities of the recombinant proteins and the *T. gondii* lysate antigen to an anti-P22 monoclonal antibody (MAb) and human sera were determined by immunoblot analysis as described previously (11). For the immunoblot analysis with anti-P22 MAb 87-21-6D10 (11), an immunoblot of *T. gondii* lysate antigen, rP22 purified protein, v22 fusion protein, and GST wild-type protein was incubated with culture supernatants of hybridoma 87-21-6D10 or Sp2/0 control cells.

For immunoblot analyses with human sera, immunoblots of reduced and nonreduced v22 fusion protein, GST wild-type protein, and *T. gondii* lysate antigen were incubated with sera that were diluted 1:100 in PBS–0.05% Tween 20 (PBS-T) containing 5% nonfat dry milk (7). The conjugates used were HRPO-conjugated goat anti-human IgA, IgG, or IgM antibodies that were used at previously determined optimal dilutions (1:750, 1:3,000, and 1:4,000, respectively) in PBS-T containing 3% bovine serum albumin. The substrate was 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and was used at a final concentration of 0.1 mg/ml in PBS. Hydrogen peroxide was used at 0.15%. Control immunoblots performed to test for the reactivities of the conjugates to v22 fusion protein, GST wild-type protein, or *T. gondii* lysate antigen did not reveal any bands (data not shown).

**Serum samples.** Sera were collected from 88 patients; 31 had serologic evidence of recently acquired *T. gondii* infection (2), 31 had serologic evidence of chronic infection (they were seropositive for *T. gondii* antibodies for at least 2

years), and 26 were seronegative for *T. gondii* antibodies. Sera were tested by the Sabin-Feldman dye test (DT) (5), the double-sandwich IgM ELISA (DS-IgM ELISA) (10), the DS-IgA ELISA (16), and the AC/HS test (acetone-fixed tachyzoites/formalin-fixed tachyzoites) (2). The sera from patients with recently acquired *T. gondii* infection had DT titers that ranged from 1:1,024 to 1:32,000, DS-IgM ELISA titers that ranged from 1.8 to 9.9, DS-IgA ELISA titers that ranged from 1 to >22.4, AC titers that were ≥1,600, and HS titers that ranged from 1,600 to ≥3,200. Chronically infected patients had DT titers that ranged from 1:32 to 1:512, DS-IgM ELISA titers that ranged from 0 to 4.7, DS-IgA ELISA titers that ranged from 0 to 0.8, AC titers that were <100, and HS titers that ranged from 100 to 800. The classification of acute or chronic infection was based on the individual's clinical history as well as the serological test results (2, 13). A pool of serum samples from six seronegative individuals, each of whom was negative when their serum samples were tested undiluted by DT, was used as a control for the immunoblots and the P22 ELISA.

**P22 ELISA.** Each well of a microtiter plate (Nunc, Roskilde, Denmark) was coated with 10 μg of v22 fusion protein, GST wild-type protein, or *T. gondii* tachyzoite lysate antigen in 1 ml of 0.05 M carbonate buffer (pH 9.6) or with carbonate buffer only. In preliminary experiments, 10 μg of v22 fusion protein or GST wild-type protein per ml was determined to be the optimal concentration with which to coat the wells of the ELISA plates. After incubation for 1 h at 37°C, the plates were washed three times with PBS-T and postcoated with 200 μl of 5% bovine serum albumin in PBS-T per well for 2 h at 37°C. The plates were then washed as described above, and 100 μl of test or control serum diluted 1:100 in 1% bovine serum albumin in PBS-T was applied to each well with v22 fusion protein, GST wild-type protein, or no antigen. The plates were incubated overnight at 4°C and washed as described above; and then each well received 100 μl of HRPO-conjugated goat anti-human IgA, IgG, or IgM at 1:500, 1:1,000 and 1:4,000, respectively. The plates were incubated for 1 h at 37°C and washed as described above, and then 100 μl of 0.03% *o*-phenylenediamine (Sigma) was added to each well. The optical density values were measured with an automatic ELISA reader (Dynatech Laboratories, Chantilly, Va.) after 15 min of incubation at room temperature. Each sample was run in duplicate wells. The ELISA results were determined for each patient by taking the mean value of the two absorbance readings with the v22 fusion protein and then subtracting the mean value of the two absorbance readings with the GST wild-type protein. A sample was considered positive if the calculated absorbance value was greater than the mean ± 1 standard deviation for the 26 normal individuals.

## RESULTS

**Expression of full-length P22 gene.** The essentially full-length cDNA insert c88 (11) which encodes amino acids 17 to 186 of the P22 gene was subcloned into the pGEX-2T expression system (15) in order to generate a fusion to GST and allow the purification of the fusion protein by affinity to glutathione agarose. To subclone the c88 *Eco*RI insert into pGEX-2T and maintain the same reading frame as in λgt11, a 24-bp adapter was placed in front of the insert. The resulting recombinant, pGEX-2T-c88, was found to express a fusion protein of approximately 48 kDa, as expected, but the protein could not be purified by affinity to glutathione agarose because of insolubility and it reacted poorly with

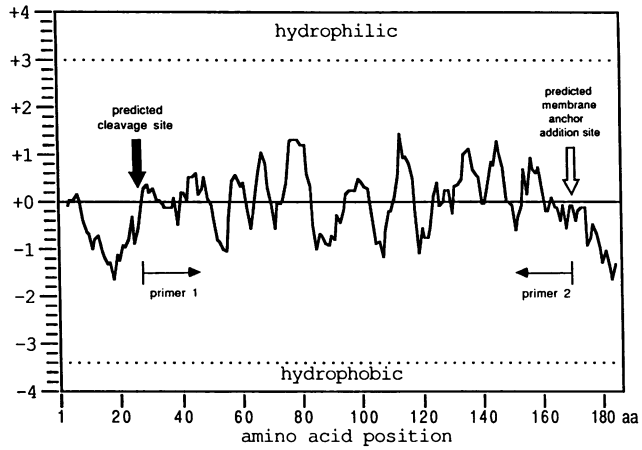


FIG. 1. Hydropathicity plot of predicted P22 amino acid sequence. The entire open reading frame of P22 was plotted by using the hydrophilicity parameters of Hopp and Woods (6) on DNA Inspector IIe software (Textco, Lebanon, N.H.), with an averaging length of six amino acids. The theoretical sites for signal peptide cleavage (bold arrow) and removal of 14 C-terminal amino acids during attachment of a glycolipid membrane anchor (open arrow) are shown. The region of the gene amplified by PCR with primers 1 and 2 is indicated.

two anti-P22 MAbs, 87-21-6D10 and 87-21-5A6 (11), on immunoblots of whole recombinant bacterial lysate (data not shown).

**Construction of recombinant P22 on the basis of the predicted mature polypeptide by using PCR.** In our previous study (11), we determined that the precursor P22 protein in *T. gondii* is encoded by an open reading frame of 558 bp with a predicted primary translation product of 186 amino acids. The sequence was found to contain a putative signal sequence of 26 residues and an approximate 14-amino-acid C-terminal domain believed to be cleaved during addition of the glycolipid membrane anchor (20). Thus, after processing with signal peptidase and the addition of the membrane anchor, the mature polypeptide was predicted to be about 146 amino acids long, perhaps encompassing residues 27 to 172. We found that both the predicted signal peptide and the C-terminal domain were markedly hydrophobic, as illustrated by the hydrophobicity analysis of Hopp and Woods (6) (Fig. 1), and suspected that this might account for the insolubility of the pGEX-2T-c88 fusion protein. To eliminate these domains and create a recombinant protein that more closely resembles the predicted mature protein, PCR was used to amplify a 438-bp region of the P22 gene encoding amino acids 27 to 172. Oligonucleotides carrying *Bam*HI and *Eco*RI sites were designed to amplify this region, as shown in Fig. 1. The 446-bp insert was spliced into pGEX-2T, creating a fusion product with GST, and was designated the v22 fusion protein. The v22 fusion protein was found to be approximately 45 kDa and could be expressed in abundance upon induction of bacterial cultures (Fig. 2, lane 2). The fusion protein was released from the cells following sonication, and a large proportion was found in the soluble fraction (lane 3). The fusion protein was readily purified by affinity to glutathione agarose (lane 4) and could be cleaved with thrombin to release rP22 and the GST fusion partner (lane 5). In addition to amino acids 27 to 172 from the P22 gene, the released rP22 protein carried an N-terminal glycine residue from the thrombin site that was retained after cleavage. The

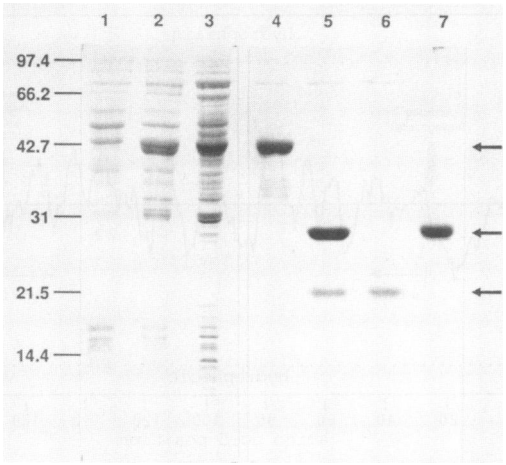


FIG. 2. Expression and purification of rP22. Samples of uninduced bacterial culture (lane 1), culture induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h (lane 2), supernatant after sonication and centrifugation (lane 3), purified v22 fusion protein (lane 4), thrombin-cleaved v22 fusion protein (lane 5), purified rP22 (lane 6), and purified GST wild-type protein (lane 7) were fractionated by electrophoresis on a 10 to 15% acrylamide gradient gel and stained with Coomassie brilliant blue (9). The arrows show the positions of v22 fusion protein (upper), GST wild-type protein (middle), or rP22 (lower). The numbers at the left indicate the sizes (in kilodaltons) of the protein standards.

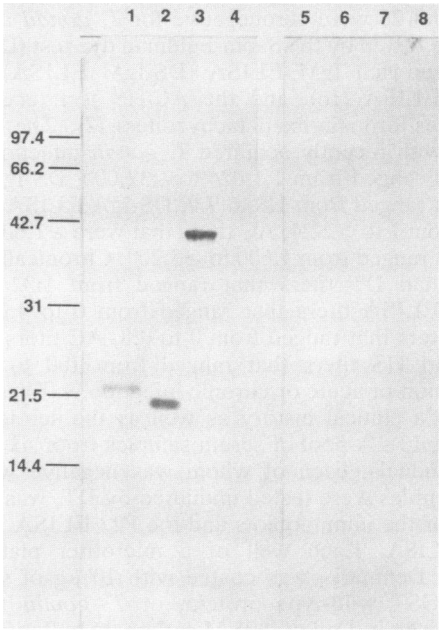


FIG. 3. Immunoblot analysis of fusion proteins and *T. gondii* lysate antigen by using anti-P22 MAb 87-21-6D10. About 20 μg of protein of RH tachyzoite lysate (lanes 1 and 5), 5 μg of purified rP22 (lanes 2 and 6), 5 μg of v22 fusion protein (lanes 3 and 7), and 5 μg of GST wild-type protein (lanes 4 and 8) were fractionated by electrophoresis on a 10 to 15% acrylamide gradient gel, transferred to nitrocellulose, and probed with either 87-21-6D10 culture supernatant (lanes 1 to 4) or control Sp2/0 culture supernatant (lanes 5 to 8) as described previously (11). Following antibody incubations, blots were developed by reaction with HRPO-conjugated goat anti-mouse IgG. Numbers at the left indicate the sizes (in kilodaltons) of the protein standards.

published procedure for eliminating the GST partner after thrombin cleavage by repeated passage over glutathione agarose (15) was found to be ineffective for purifying rP22. We found that thrombin cleavage of the v22 fusion protein while it was still bound to glutathione agarose was much more effective at recovering rP22 without the contaminating GST partner (lane 6).

**Reactivity of P22 MAb to v22 fusion protein and rP22.** In immunoblot analyses, the anti-P22 MAb 87-21-6D10 reacted specifically with native P22 in *T. gondii* lysate antigen (Fig. 3, lane 1), purified rP22 (lane 2), and v22 fusion protein (lane 3); but it did not react with the GST wild-type protein (lane 4). A similar reaction was observed with the anti-P22 MAb 87-21-5A6 (data not shown). Furthermore, rP22 migrated near the 21.5-kDa marker and only slightly faster than native P22 on polyacrylamide gels, even though the theoretical molecular mass of rP22 is only about 15 kDa. There was no detectable reaction against these same proteins when the Sp2/0 control culture supernatant was used (lanes 5 to 8).

**Reactivity of human serum to v22 fusion protein on immunoblots.** Ten serum samples were tested for the presence of IgG, IgA, and IgM antibodies to P22 by reaction to immunoblots of the v22 fusion protein; five serum samples from patients with recently acquired infection and five serum samples from chronically infected patients were tested. Examples from each group of immunoblots by using the reduced v22 fusion protein as antigen are shown in Fig. 4. Antibodies of the IgG and IgA classes which reacted to the v22 fusion protein were detectable; the bands in sera from five of five patients with recently acquired infection as well as from five of five chronically infected individuals had various intensities, although in general there was a stronger reaction in the samples from acutely infected individuals. Antibodies of the IgM class which reacted to v22 fusion protein were detectable only in sera from three of five patients with recently acquired infection, and they were not detectable in any of the chronically infected individuals. Immunoblots with nonreduced v22 fusion protein produced similar results (data not shown).

When immunoblots of the reduced and nonreduced GST wild-type protein were reacted with the same 10 serum samples from infected individuals, bands with slightly greater intensities than those observed with the negative control sera were noted in sera from only two of the patients with recently acquired infections, although the reaction was markedly less than the reaction to the v22 fusion protein (data not shown). Serum from one of the latter two patients had a detectable reaction to the GST wild-type protein in the IgG immunoblot, while serum from the other patient reacted weakly with the GST wild-type protein in both IgA and IgG immunoblots (data not shown). Since IgG antibodies from all 10 serum samples reacted with the v22 fusion protein, control immunoblots were performed to ascertain whether the IgG antibodies present in the 10 serum samples recognized native P22. All sera revealed a band of variable intensity with an approximate molecular mass of 22 kDa in immunoblots of reduced and nonreduced *T. gondii* lysate (data not shown).

**Reactivity of human serum to v22 fusion protein in ELISA. (i) IgG ELISA.** Two separate IgG ELISA experiments were performed. In the first, the 10 serum samples used for immunoblots plus an additional 8 serum samples from acutely infected patients and 8 serum samples from chronically infected patients were tested in an IgG ELISA with the v22 fusion protein; 13 of 13 serum samples from the acutely

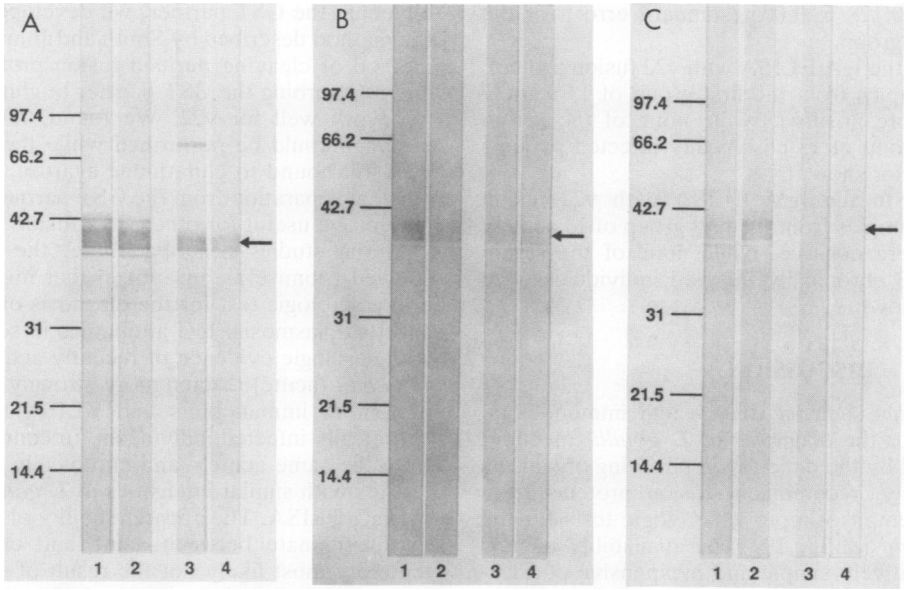


FIG. 4. Immunoblot analyses of v22 fusion protein reacted with sera from infected patients. About 225 μg of reduced v22 fusion protein was loaded across a 10 to 15% acrylamide gradient gel, fractionated by SDS-PAGE, transferred to nitrocellulose (11), and probed with sera from two patients with recently acquired *T. gondii* infection (lanes 1, DT = 1:8,000; DS-IgM ELISA = 4.9; lanes 2, DT = 1:8,000, DS-IgM ELISA = 5.7) and sera from two patients with chronic *T. gondii* infection (lanes 3, DT = 1:32; DS-IgM ELISA = 0; lanes 4, DT = 1:32; DS-IgM ELISA = 0.9). Following antibody incubations, strips were developed by reaction with HRPO-labeled goat anti-human IgG (A), goat anti-human IgA (B), and goat anti-human IgM (C) antibodies. The arrows show the positions of the v22 fusion protein. The numbers at the left indicate the sizes (in kilodaltons) of the protein standards.

infected individuals and 11 of 13 serum samples from the chronically infected individuals were positive. There was a marked difference in the absorbance values between sera from the two groups (Fig. 5A); the mean value for the acutely infected group was 0.214 ± 0.015 (standard error), while the mean value for the chronically infected group

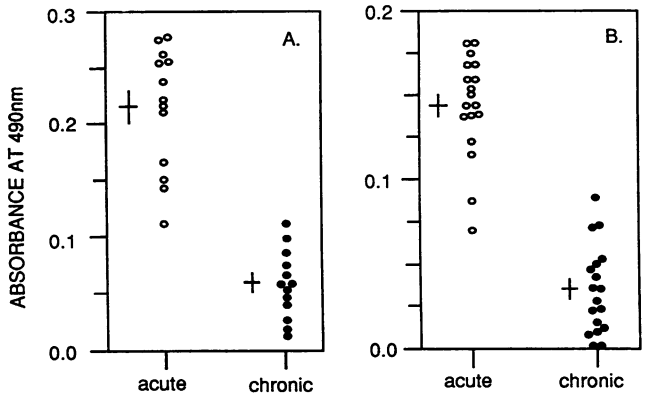


FIG. 5. Absorbance values of IgG antibodies present in sera from acutely and chronically infected individuals which recognize v22 fusion protein determined by ELISA. Experiment 1 (A) was performed with sera from 13 acutely and 13 chronically infected individuals, while experiment 2 (B) was performed with a second set of sera from 18 acutely infected and 18 chronically infected individuals. Wells were coated with v22 fusion protein or GST wild-type protein. The serum samples that were tested are described in the text and were diluted 1:100. Values are mean calculated absorbances determined as described in the text. The means (horizontal bars) ± standard errors (vertical bars) are indicated for each group.

was 0.058 ± 0.008 (standard error). In contrast, when the same 24 serum samples were reacted to *T. gondii* lysate antigen in an IgG ELISA, there was no obvious difference in the absorbance values between the two groups; the mean value for the acutely infected group was 0.148 ± 0.007 (standard error), while the mean value for the chronically infected group was 0.146 ± 0.005 (standard error). Three of the serum samples from acutely infected patients, the three acutely infected patients whose sera had the lowest absorbance values in Fig. 5A, reacted with the GST wild-type protein which may account for the low positive results, since in our determination of the absorbance value, the absorbance for the GST wild-type protein was subtracted from the absorbance for the v22 fusion protein. Two of the serum samples from the chronically infected patients had absorbance values within the mean ± 1 standard deviation (0.003 ± 0.018) of the negative control group and were considered negative. None of the samples from chronically infected patients had significant background reactivity with the GST wild-type protein that would affect the determination of the ELISA absorbance values.

The second experiment with sera from different individuals than those used in the first experiment revealed that 18 of 18 serum samples from the acutely infected patients and 12 of 18 serum samples from the chronically infected patients were positive. Again, there was a marked difference in the absorbance values between the two groups (Fig. 5B); the mean value for the acutely infected group was 0.144 ± 0.007 (standard error), while the mean value for the chronically infected group was only 0.034 ± 0.006 (standard error). In contrast, the mean absorbance values for the two groups against *T. gondii* lysate antigen in an IgG ELISA were similar: 0.121 ± 0.004 (standard error) for the acutely

infected group and 0.128 ± 0.005 (standard error) for the chronically infected group.

**(ii) IgA ELISA.** In the IgA ELISA with v22 fusion protein, only two serum samples from the first group of 13 acutely infected patients were positive, while none of the serum samples from the group of 13 chronically infected patients were positive (data not shown).

**(iii) IgM ELISA.** In the IgM ELISA with v22 fusion protein, six serum samples from the first group of 13 acutely infected patients were positive, while none of the serum samples from the 13 chronically infected individuals were positive (data not shown).

## DISCUSSION

Serodiagnostic tools such as ELISA and immunoblots, although effective for the diagnosis of *T. gondii* infection, have been hampered by the difficulty in purifying organisms in quantity. Recently, recombinant fusion proteins have been used in an attempt to improve serologic tests for the diagnosis of *T. gondii* (8, 18, 19). The availability of new methods for the relatively simple and inexpensive purification of recombinant antigens such as pGEX-2T should allow for more accurate standardization of serologic tests.

Cell surface proteins of protozoan parasites have been the focus of extensive research for their potential as diagnostic antigens since they are accessible to the immune system of the host from the beginning of the infection. The gene that encodes surface antigen P22 of *T. gondii*, one of the four major surface antigens of the invasive tachyzoite form (1, 4), was recently isolated in our laboratory and was therefore chosen for further analysis to determine the diagnostic potential of recombinant fusion proteins carrying antigenic portions of this protein.

Because of the inability to purify the P22–β-galactosidase fusion protein, the recombinant protein described in our earlier report (11), we attempted to produce and purify an rP22 as a fusion to GST by using the pGEX-2T expression system, which allows soluble GST fusion proteins to be easily purified by affinity to glutathione agarose. However, the GST fusion protein made from the original λgt11 cDNA insert c88 was found to be insoluble and could not be purified. In addition, the fusion protein was poorly recognized by two anti-P22 MAbs. After analysis of the predicted amino acid sequence by hydrophobicity plot, we speculated that the insolubility was due to two highly hydrophobic domains that correspond to the N-terminal signal peptide and the C-terminal membrane anchor sequence. The construction by PCR technology of a truncated P22 insert that lacked these two domains yielded a P22-GST fusion protein that was soluble, could be produced in large amounts, was easy to purify, and was recognized by two anti-P22 MAbs in reduced and nonreduced immunoblots. Taken together, these facts suggest that the protein is folded in a native conformation.

After removal of the GST carrier by cleavage with thrombin, the recombinant P22 was still recognized by anti-P22 antibodies on immunoblots. Furthermore, cleaved recombinant P22 migrated on these immunoblots near the native P22 (Fig. 3), even though the calculated molecular mass of the encoded polypeptide on the gene fragment is only 15 kDa. This suggests that the apparent size of native P22 can be accounted for primarily by the anomalous mobility of the polypeptide and is probably not due to extensive modifications such as glycosylation.

To release the rP22 from the v22 fusion protein and eliminate the GST partner, we developed a modification of the method described by Smith and Johnson (15), since their method of cleaving purified fusion proteins with thrombin and readsorbing the GST partner to glutathione agarose did not work well for v22. We found, rather, that thrombin cleavage could be performed while the v22 fusion protein was still bound to glutathione agarose, resulting in a more efficient separation from the GST partner. This modification may prove useful for other GST fusion proteins as well.

In the studies described here, the v22 fusion protein showed promise as an antigen that might be incorporated into a serologic test for the diagnosis of *T. gondii* infection and toxoplasmosis. IgG antibodies in sera from individuals with serologic evidence of recently acquired infection with *T. gondii* (acute) reacted more strongly with the v22 fusion protein in immunoblots and ELISA than did sera from chronically infected individuals. In contrast, IgG antibodies from the same acutely and chronically infected individuals reacted with similar intensities to *T. gondii* lysate antigen in the IgG ELISA. The apparent ability of the P22 IgG ELISA to discriminate between acute and chronic infections is therefore most likely not the result of serendipitous differences in total antitoxoplasma IgG antibody titers. These results reveal that, in the chronically infected patients, the total antitoxoplasma IgG antibody titers measured by ELISA remained high, while the anti-P22 IgG antibody titers fell off.

The observation that DT and AC/HS titers were lower in the chronically infected group than in the recently infected group is most likely due to the fact that these tests use intact *T. gondii* organisms as the antigen, so they measure antibodies to surface antigens rather than antibodies to total parasite antigens. The fact that the DT titers, AC/HS titers, and the P22 IgG ELISA absorbances were all lower in sera from the chronically infected individuals may have been due to a generalized drop in antibody titer to surface antigens in the chronic phase of the infection.

In our preliminary studies with the ELISA, antibodies of the IgA and IgM classes did not react as well with the v22 fusion protein, despite the reactivities of both classes with *T. gondii* lysate antigen in immunoblots. The reason for this is being examined.

Because of the background reaction of human sera to GST reported by Tenter and Johnson (18), we included GST as a control antigen in the studies described here. We also observed this background reaction to GST in immunoblots and in the ELISA. Two of the serum samples from acutely infected individuals had a high level of IgG antibodies to GST; however, the reaction to v22 was noticeably stronger.

The P22 ELISA might be improved by using as the antigen recombinant P22 that has had the GST carrier removed. We described here a procedure for isolating large amounts of rP22 that are essentially free of contamination with the GST carrier. By using rP22 as an antigen for ELISA, the problems caused by the background reaction of human sera to GST might be eliminated. Further improvement might be achieved by using anti-P22 antibodies to trap rP22 on ELISA plates instead of coating the wells on ELISA plates directly with antigen. Finally, as has been suggested by Tenter and Johnson (18), these tests might be improved by the inclusion of additional recombinant antigens.

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