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A 30kD Surface Protein of *Toxoplasma gondii* as a Major Antigen for Sabin-Feldman's Dye Test

ASAO MAKIOKA[1], AKIO KOBAYASHI[1] AND KEN-ICHI HIRAOKA[2]

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Abstract

A monoclonal antibody against one of *Toxoplasma* membrane antigens was produced. The antibody of the IgM class was highly positive in both the dye test (DT) and latex agglutination test (LA) and reacted with the cellular rim of tachyzoites by immunoperoxidase staining. Western blot analysis of *Toxoplasma* membrane antigens identified the same protein with an apparent molecular weight of 30,000 (p30) as the one described previously. The p30 reacted most strongly to DT antibodies depending on the titers as compared with other membrane antigens. Also, p30 reacted more strongly with DT-positive sera than LA-positive ones, suggesting that p30 is an important antigen for DT.

Key words: *Toxoplasma gondii*, major surface protein, monoclonal antibody, dye test antigen

Introduction

The Sabin-Feldman's dye test (DT) is regarded as the most reliable method for serologic diagnosis of toxoplasmosis. However, several technical difficulties in DT largely limit the routine use. Surface membrane antigens of tachyzoites of *Toxoplasma gondii* are considered to be responsible for DT. Although no successful isolation of the surface membrane has been made, hybridoma technique enabled us to identify and isolate the membrane antigens. Several laboratories have produced monoclonal antibodies (mAb) against the surface of the tachyzoites (Handman *et al.*, 1980; Kasper *et al.*, 1983; Rodriguez *et al.*, 1985). Most of these mAb recognized a major surface protein with an apparent molecular weight of 27,000 to 30,000, which has been named p30 (Kasper *et al.*, 1983). Santoro *et al.* (1985) first used p30 in serodiagnosis of toxoplasmosis and demonstrated that all patients they tested, with acute or chronic toxoplasmosis, had significant levels of anti-p30 antibodies as assayed by ELISA.

In the present study, we also attempted to produce mAb to membrane antigens of *T. gondii* and identified one mAb generated as being directed to the major surface protein (p30) of tachyzoites. We further characterized p30 as a major antigen for DT by immunoblotting.

Materials and Methods

Monoclonal antibody

Spleen cells from female BALB/c mice immunized three times with *Toxoplasma* ghosts were fused with NS-1 myeloma cells in the presence of polyethylene glycol (Oi and Herzenberg, 1980). Antibody production was assessed by latex agglutination test (LA) using a commercial kit (Toxo-MT: Eiken Chemical Co., Tokyo). Positive hybrid cell lines were cloned twice by limiting dilution. One clone designated as 3D11 with the highest antibody titer was selected and ascites was produced in pristane-primed BALB/c mice. The monoclonal antibody (mAb) was IgM and purified by a 50% ammonium sulphate precipitation followed by a gel

[1] *Department of Parasitology, Jikei University School of Medicine, Tokyo 105, Japan*
[2] *Eiken Chemical Co., Nogi, Tochigi-ken 329-01, Japan*
牧岡朝夫　小林昭夫（東京慈恵会医科大学寄生虫学教室）
平岡謙一（栄研化学）

filtration on Sephadex G-200. Biotinylation of the mAb was made by a modification (Ogata *et al.*, 1984) of the method by Guesdon *et al.* (1979).

Antibody titration

Antibodies to *Toxoplasma* of the mAb and human sera were titrated by Sabin-Feldman's dye test (DT) and LA. The DT was performed by a modification (Kobayashi *et al.*, 1968) of the technique described by Frenkel and Jacobs (1958). The LA was performed as previously described (Kobayashi *et al.*, 1977). DT and LA titers above 1:16 and 1:32 were regarded as positive, respectively (Kobayashi *et al.*, 1977).

Localization of antigen

The localization of antigen was detected by immunoperoxidase staining using the biotinylated mAb 3D11 and horseradish peroxidase-avidin D as described by Ogata *et al.* (1984).

Preparation of Toxoplasma *membrane antigens*

Parasites were harvested from the peritoneal exudate of mice infected with the RH strain of *T. gondii*. Debris and host cells were removed by filtration through a polycarbonate membrane (pore size, 3 μm; Nuclepore Co., Pleasanton, CA). The tachyzoites were washed three times with saline by centrifugation at 900 $\times$ *g* for 5 min and disrupted by treatment with distilled water and sonication. The extract was centrifuged at 40,000 $\times$ *g* for 30 min to isolate the membrane fractions. The membrane pellets were thoroughly washed with saline by centrifugation. To solubilize the membrane antigens, 5 volumes of 0.5% Triton X-100 were added to the pellet and agitated at 4°C overnight. The resulting suspension was centrifuged at 100,000 $\times$ *g* for 30 min. The supernatant fluid was collected and used as the membrane antigens.

Rabbit antiserum to Toxoplasma *membrane antigens*

A rabbit was immunized six times at intervals of 2 weeks with 1 mg of the antigen emulsified by Freund's complete adjuvant. The rabbit was bled 2 weeks after the final injection. The IgG fraction of the antiserum was purified by passage through a protein A-Sepharose column (Pharmacia Fine Chemicals, NJ).

Electrophoresis and electophoretic transfer

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Tris-glycine buffer, pH 8.3, containing 0.1% SDS as described by Laemmli (1970) on 15% polyacrylamide slab gels. Transfer of separated antigens on the SDS gel to a nitrocellulose sheet was carried out in 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, with a commercial blotting apparatus (Atto Co., Tokyo).

Immunoblotting

The mAb antigen was detected on the nitrocellulose sheet by the avidin-biotin system using biotinylated mAb as described by Ogata *et al.* (1984). For the immunoblotting with human antisera the technique described by Partanen *et al.* (1983) was used. Heavy chain-specific, peroxidase conjugated rabbit anti-human IgG (Cappel laboratories, PA) was used.

Radioiodination of Toxoplasma *surface proteins*

Purified tachyzoites were subjected to ^{125}I surface-labelling by IODOGEN method as described by Kasper *et al.* (1983). The radioiodinated tachyzoites were washed three times in phosphate buffered saline, pH 7.2 (PBS) by centrifugation at 900 $\times$ *g* for 5 min, and the final pellet was dissolved in 0.5% Nonidet P40 (Nakarai Chemical Co., Kyoto) and allowed to lyse for 2 h at 4°C. The lysate was centrifuged at 12,800 $\times$ *g* for 5 min, and the supernatant fluid was stored at -20°C for subsequent analysis.

Immunoprecipitation

Radioiodinated samples were treated with rabbit IgG to membrane antigens, and the bound proteins were absorbed onto protein A-Sepharose as described by Kasper *et al.* (1982). The resulting precipitate was collected and washed by centrifugation and released by dissolving the complex in the SDS-containing buffer. After electrophoresis, the gel was stained with Coomassie brilliant blue, destained, dried and autoradio-

graphed with Fuji X-ray film (Fuji Photo Film Co., Tokyo) and Du Pont Cronex Lightning-Plus intensifying screens.

Results

Immunological characterization of mAb and its antigen

One hybrid clone secreting antibody of the highest anti-*Toxoplasma* titer was chosen and designated 3D11. This antibody belonged to the IgM class and the results of its serological characterization are shown in Table 1. The antibody gave high titers in both DT and LA. The immunoperoxidase technique in which biotinylated mAb and horseradish peroxidase-avidin D are used was employed to observe the localization of 3D11 antigen. The 3D11 antibody stained the cellular rim of the tachyzoites as compared with control (Fig. 1).

Table 1. Serological characterization of a monoclonal antibody to *Toxoplasma gondii*

		Reciprocal titer	
Clone	Class	DT	LA
3D11	IgM	4,096	16,384

Monoclonal antibody was adjusted to 0.5 mg protein/ml before the assay.
DT, dye test.
LA, latex agglutination test.

Toxoplasm membrane antigens separated on SDS-PAGE were electrophoretically transferred to a nitrocellulose sheet. Antigens on the nitrocellulose sheet were detected by an immunoenzymatic technique using 3D11. *Toxoplasma* membrane antigens stained with Coomassie blue on the gel are shown in Fig. 2A. The band of molecular weight 30,000 was most intensely stained. The 3D11 antibody recognized a single antigen with an apparent molecular weight of 30,000 (Fig. 2C) as compared with control (Fig. 2B). Presence of 3D11 antigen in the surface membrane of the tachyzoites was further confirmed by immunoprecipitation. Radioiodinated surface proteins were precipitated by rabbit IgG to *Toxoplasma* membrane antigens. The complex was absorbed onto protein A-Sepharose, released and analysed by SDS-PAGE and autoradiography. As shown in Fig. 3, the antibody precipitated predominantly the band of molecular weight 30,000. No band was precipitated by nonimmune rabbit IgG (data not shown). From these results we confirmed that 3D11 antibody was directed to a surface membrane protein with an apparent molecular weight of 30,000 (p30) as previously described (Handman *et al.*, 1980; Kasper *et al.*, 1983; Rodriguez *et al.*, 1985; Santoro *et al.*, 1985).

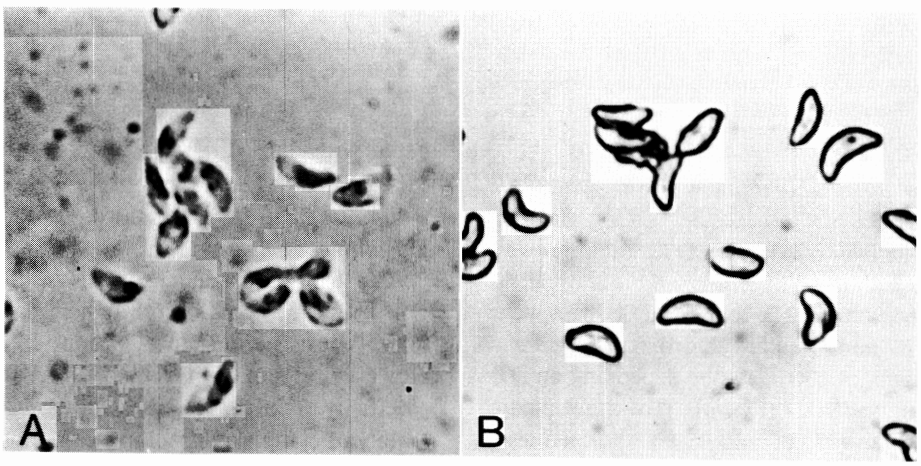

Fig. 1. Localization of 3D11 antigen by immunoperoxidase staining, using biotinylated 3D11 and horseradish peroxidase-avidin D. A, control; B, biotinylated 3D11.

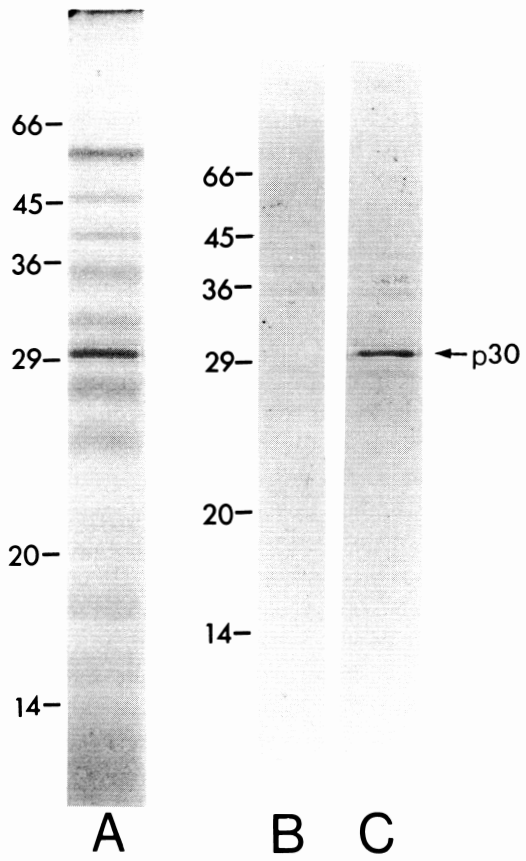

Fig. 2. Immunochemical detection of 3D11 antigen on nitrocellulose strips by biotinylated 3D11 and horseradish peroxidase-avidin D. A, *Toxoplasma* membrane antigens; B, control strip treated with unbiotinylated 3D11; C, biotinylted 3D11. The p30 shows a protein with molecular weight of 30,000. Numbers represent molecular weight standards: bovine albumin (66,000), egg albumin (45,000), glyceraldehyde dehydrogenase (36,000), carbonic anhydrase (29,000), trypsin inhibitor (20,100), and lactoalbumin (14,200).

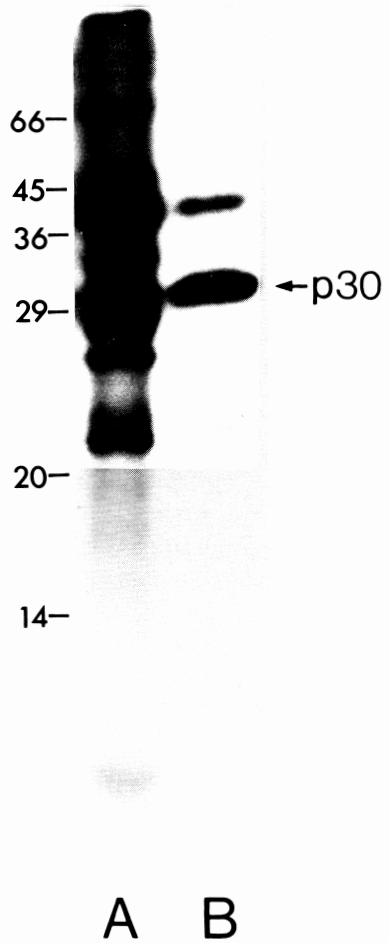

Fig. 3. Presence of 3D11 antigen in ^{125}I-labeled surface membrane antigens of *Toxoplasma* tachyzoites. A, *Toxoplasma*-radioiodinated surface antigens; B, antigens immunoprecipitated by rabbit IgG to membrane antigens. The p30 is a protein of m.w. 30,000 and numbers are m.w. standards.

Reactivity of 3D11 antigen (p30) with DT antibodies

The reactivity of p30 with DT antibodies was compared with those of other membrane antigens by immunoblotting. The nitrocellulose strips were treated with human sera with different DT titers. The reactivity of p30 increased depending on DT titers from 4 to 1024 (Fig. 4). Two other membrane proteins (58,000 and 35,000 daltons) also reacted with DT antibody but their reactivities were not correlated with the DT titers as compared with that of p30.

Amount of p30 in Toxoplasma *membrane and LA antigens*

The LA kit product (Eiken Chemical Co., Tokyo) is simple and useful for serodiagnosis of toxoplasmosis and most commonly used. However, it is still inferior to DT in regard to specificity. The antigens used for the LA consist mainly of cytoplasmic, soluble components. We compared relative amount of p30 between the *Toxoplasma* membrane antigens and antigens for

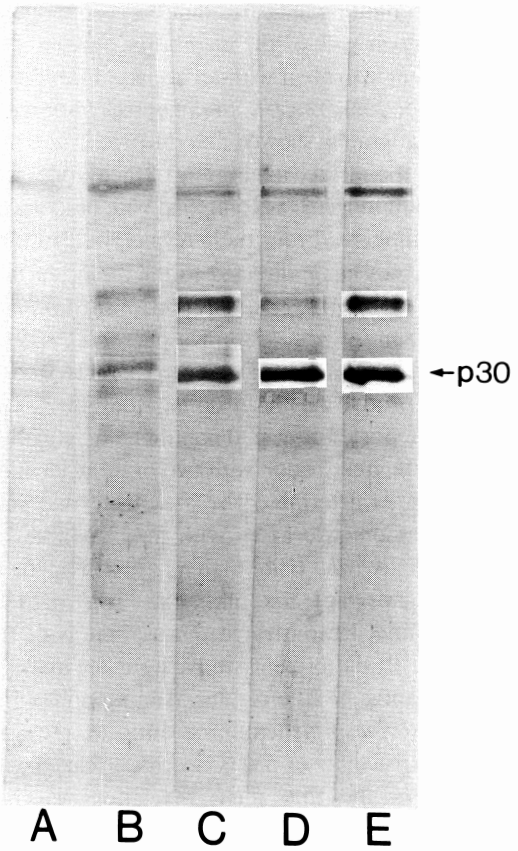

Fig. 4. Reactivity of 3D11 antigen (p30) with dye test (DT) antibodies. The strips of *Toxoplasma* membrane antigens were treated with various human antisera diluted to 1:400 and with peroxidase conjugated rabbit anti-human IgG diluted to 1:400. A, no serum; B, serum with DT 4 and LA 128; C, serum with DT 64 and LA 128; D, serum with DT 256 and LA 128; E. serum with DT 1024 and LA 128. The p30 is a protein of m.w. 30,000 and numbers are m.w. standards.

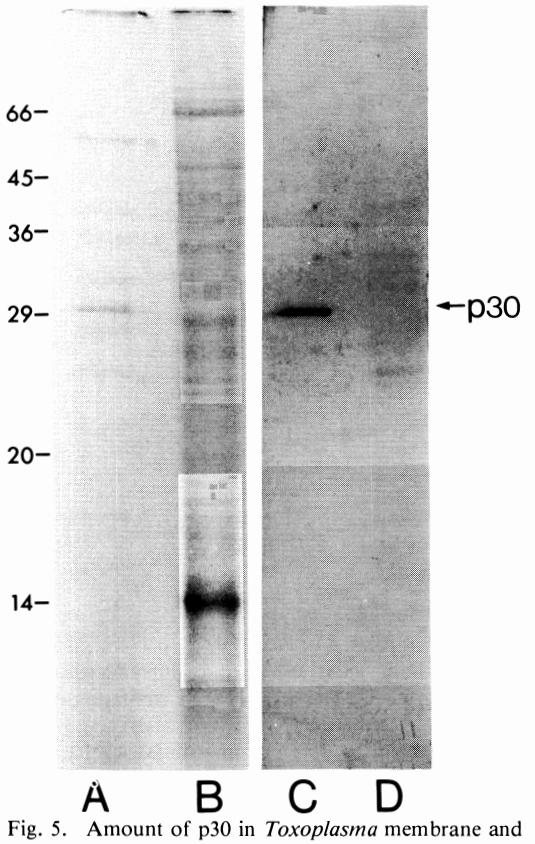

Fig. 5. Amount of p30 in *Toxoplasma* membrane and LA antigens. The membrane antigens and LA antigens were electrophoresed in equal concentration of protein and transferred to a nitrocellulose sheet. The strips were then treated by biotinylated 3D11 antibody and p30 was detected by avidin-biotin system as described in Fig. 2. A, *Toxoplasma* membrane antigens; B, *Toxoplasma* antigens for LA: C, membrane antigen strip treated with biotinylated 3D11: D, LA antigen strip treated with biotinylated 3D11. The p30 is a protein of m.w. 30,000 and numbers are m.w. standards.

LA. For this purpose, the membrane antigens and LA antigens were electrophoresed and transferred to a nitrocellulose sheet. The strips were then treated with biotinylated 3D11 antibody and p30 was detected by avidin-biotin system. Coomassie blue stained bands of both antigens are shown in Fig. 5, A and B. The band of p30 was overlapped by the LA antigens. Immunoblotting with 3D11 antibody showed that p30 was detected in the membrane antigens but not in the antigens for LA (Fig. 5, C and D). The finding suggests that the amount of p30 involved in the LA antigens is very small, if any, compared with that in the membrane antigens.

Reactivity of p30 with DT-positive sera

The reactivity of p30 was compared between DT-positive sera and LA-positive ones by immunoblotting. The strips were treated with human sera with different DT and LA titers. The results are shown in Fig. 6. Serum which was negative by both DT and LA did not react to p30.

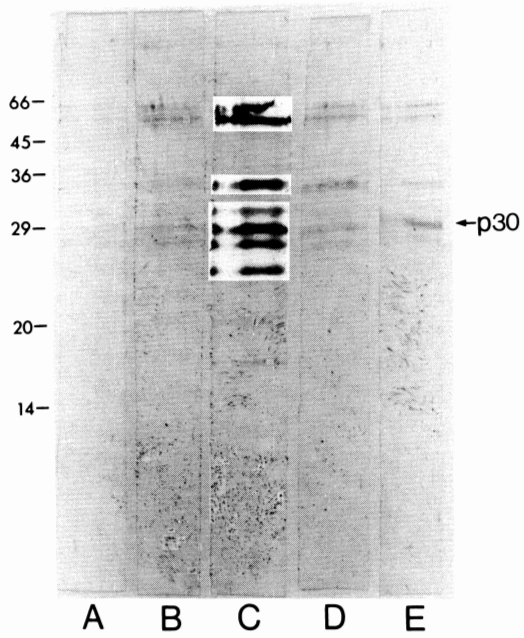

Fig. 6. Comparison of reactivity of p30 with dye test (DT)-positive sera and latex agglutination test (LA)-positive ones. The strips of the membrane antigens were treated with (A) no serum, (B) serum with DT 4 and LA 2, (C) serum with DT 4096 and LA 1024, (D) serum with DT 4 and LA 32, and (E) serum with DT 16 and LA 16. The p30 is a protein of m.w. 30,000 and numbers are m.w. standards.

Serum with positive DT and LA reacted strongly to the molecule. When serum showing positive DT but negative LA was compared with one which was negative in DT but positive in LA, the former reacted more strongly with p30 than the latter.

Discussion

We have generated a mAb to *Toxoplasma gondii* and the corresponding antigen was characterized in the present paper. The results indicated that the mAb was highly positive by DT and that the antibody stained the cellular rim of the parasite. This suggests that the antibody is directed to the surface membrane of tachyzoites and also that the corresponding antigen may play an important role in the protective immune response against *T. gondii*. The results of Western blotting indicated that the corresponding antigen was part of the membrane antigens and was a single protein with an apparent molecular weight of 30,000. Also, immunoprecipitation and autoradiography showed that the protein was present on the surface membrane of tachyzoites and most immunogenic as compared with other membrane antigens. From these results we identified the mAb as being directed to the major surface protein (p30) of *T. gondii* as previously described (Handman *et al.*, 1980; Kasper *et al.*, 1983; Rodriguez *et al.*, 1985). Some characterization of this protein has been done. Rodriguez *et al.* (1985) reported that p30 contained a single immunodominant region with two or more identical epitopes as determined by a two-site/one antibody radiometric assay with mAb. Moreover, Santoro *et al.* (1985) first applied p30 to serodiagnosis of toxoplasmosis using ELISA system and demonstrated the usefulness of the protein. In the present study, we evaluated p30 as DT antigen. The results of immunoblotting wih *Toxoplasma* membrane antigens revealed that the reactivity of p30 is closely correlated with DT titers and that such correlation was not observed in other membrane antigens. This suggests that p30 is the most important antigen for DT. In this regard, Handman *et al.* (1980) reported that sera from humans with acute infection showed a wider variability of the relative intensity of the membrane antigens and that one serum out of five sera they tested did not precipitate p30. Since they did not describe DT titers of those sera, the relation between negative recognition of p30 and DT titers is unclear.

Ogata *et al.* (1984) generated three mAb that directed to 43,000 dalton antigens by immunization with the insoluble fractions of *T.gondii*. These p43 antigens were found to be involved in some insoluble organelles but not on the surface of the parasite.

The LA is a simple and useful method of serodiagnosis of toxoplasmosis but is inferior to DT in specificity. The results indicated that very little amount of p30 is involved in the antigens for LA. Also, the comparison between DT-positive and LA-positive sera indicated differences in reactivity of p30 between the two;

DT-positive sera reacted more strongly with p30 than LA-positive ones. Although there exists difference in complement dependency between DT and LA, it is conceivable that the use of p30 to sensitize latex particles would imply a possibility of improvement of the LA.

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