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Membrane Proteins and Their Antigenicity of *Toxoplasma gondii**

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Abstract: Surface membrane proteins of virulent RH strain and tissue cyst-forming Fukaya strain of *Toxoplasma gondii* were analyzed by SDS-polyacrylamide gel electrophoresis after LPO-catalyzed surface iodination and lectin blotting, then identified the zoite-specific antigens. Prior to the analyses, purification of RH tachyzoites from mouse peritoneal exudate and of Fukaya bradyzoites from mouse brain tissues were performed by centrifugation on the discontinuous Percoll density-gradient. Tachyzoites were obtained at the interface of 50% and 60% Percoll solution and brain cysts were harvested at the interfaces of 40-50% and 50-60%, then bradyzoites were obtained by treating the cysts with hypertonic solution.

The LPO-catalyzed iodination detected 15 KDa and 14 KDa proteins of bradyzoites and 30 KDa protein of tachyzoites as major bands with several other minor bands. But Con A blotting revealed some bands of 200 K~50 KDa glycoproteins of bradyzoites and 52 KDa band as major and minor bands of 33 K~20 KDa of tachyzoites. Phytohemagglutinin did not detect any band in the two forms. EITB with anti-Fukaya antibody and anti-RH antibody revealed cross-reactivities between the two forms. Despite the cross-reactivity, anti-Fukaya antibody reacted with 15 KDa band of bradyzoites specifically and, anti-RH antibody with 52 KDa, 30 KDa, and 25 KDa bands of tachyzoites, respectively.

It was identified that 15 KDa protein in bradyzoite, which was not a glycoprotein, was a major membrane protein with sufficient antigenicity, and in the case of tachyzoite, 52 KDa surface glycoprotein (gp52) with specific antigenicity might be added to the major surface protein, p30.

Key words: *Toxoplasma gondii*, biochemistry, membrane proteins, antigenicity, glycoprotein

INTRODUCTION

Toxoplasma gondii is a ubiquitous protozoan parasite that can cause stillbirth or abortion in humans and domestic animals if first contracted during pregnancy (Levine, 1977). However, since it is an obligatory intracellular parasite and therefore difficult to purify, we have little information on its protein composition or antigenic property.

The biochemical analysis of surface antigens of the invasive stage of *Toxoplasma* has been pioneered by Handman *et al.* (1980) who gave the first description of major surface proteins identified by immune sera and monoclonal antibodies. Additional data were obtained by

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several groups who published detailed analyses of some of the surface antigens (Kasper *et al.*, 1982, 1983, 1984 & 1985; Kasper, 1987; Johnson *et al.*, 1983; Dubremetz *et al.*, 1985; Rodriguez *et al.*, 1985; Couvreur *et al.*, 1988). In addition to the above data, we characterized and compared the surface antigens of RH strain (Sabin, 1941), asexual dividing tachyzoite form, and tissue cyst forming (Dubey and Frenkel, 1976) Fukaya strain in this study. Characterization and isolation of *Toxoplasma* antigens would be valuable for the study of immune response to the parasite and for the development of newer diagnostic methods and vaccines.

MATERIALS AND METHODS

1. Parasites

Two strains of *Toxoplasma* have been used in this study: RH and Fukaya. RH strain was maintained routinely by serial passage in the peritoneal cavity of ICR mice, and Fukaya strain was passaged every month by intraperitoneal injection of cysts collected from the brains of infected ICR mice. To obtain pure tachyzoites of RH strain from peritoneal exudate, they were first incubated with 1×10^{-5}M formyl-methionyl-leucyl-phenylalanine (FMLP) for 15 min at 37°C, then centrifuged over the 40, 50 and 60% Percoll (Pharmacia Fine Chemicals) density gradient at 2,000 rpm (Beckman, TJ-6) for 30 min. Tachyzoites were obtained at the interface of 50% and 60% Percoll solution. Bradyzoites of Fukaya strain were also obtained by the Percoll density gradient centrifugation. Mouse brain infected previously with Fukaya strain was homogenized with mortar and pestle, and incubated with 10,000 unit/ml deoxyribonuclease I (Sigma Chemical Co.). They were suspended with 35% Percoll solution, then centrifuged at 1,600 rpm for 30 min. The precipitates were saved and overlaid on the 40, 50, 60 and 70% Percoll density gradients. After centrifugation at 2,000 rpm for 30 min, the interfaces of 40-50% and 50-60% were removed, and then treated with hypertonic solution.

2. Immune Rabbit Sera

Tachyzoites of RH strain and bradyzoites of Fukaya strain obtained by the Percoll density gradient centrifugation were ultrasonified three times for 15 sec in Sonic dismembrator (Fisher, Model 300), and the solutions were centifuged at 12,000 rpm for 10 min. The supernatants were mixed with Freund's complete adjuvant (Difco), then injected to rabbits intradermally with the concentration of 200 μg/ml. After 1 week, second injection was performed and antibodies were produced for 10 days. The titers of antibodies were 1 : 4, and sera were partially purified by 35 to 50% ammonium sulfate precipitation.

3. Membrane Iodination

The procedure was performed according to the method of Thorell and Johansson (1971). The reactants were added rapidly in the following order and amounts : 1 mCi Na ^{125}I (New England Nuclear Co.), parasites to be labelled, 5 μg lactoperoxidase(LPO), and 1 μl of 0.88 mM H_2O_2. The reactants were mixed for 1~2 sec after which the reaction was stopped by dilution with 500 μl phosphate buffer. To remove unreacted iodide, the contents were immediately centrifuged at 12,000 rpm for 5 min.

4. SDS-Gel Electrophoresis and Western Blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli(1970) on 10% gels.

The lectins used in this study, concanavalin A (Con A) and phytohemagglutinin (PHA) were obtained from Sigma Chemical Co.. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Cappel, aliquoted, and stored at −70°C until use. ^{125}I-Con A and ^{125}I-PHA were prepared by chloramine T oxidation and purified on Sephadex G-50 as described by Chang and Cuatrecasas(1976).

Western blots were performed using the whole antigens resolved on SDS-PAGE and electrophoretically transferred to nitrocellulose (NC) paper (Hoefer Scientific Instruments) according to the procedure described by Towbin

et al. (1979). NC paper was quenched for 1 hr at room temperature with PBS containing 2% periodic acid-treated bovine serum albumin (P-ESA) (Glass *et al.*, 1981), 0.3% Tween-20, and then washed twice with 100 ml of 0.1% Tween-20 in PBS (PBS-Tween). Con A and PHA blots were carried out using ^{125}I-Con A and ^{125}I-PHA (5×10^5 cpm/ml in PBS-Tween, 1% P-BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, respectively). After incubation for 1 hr, the NC papers were extensively washed with PBS-Tween, then dried and exposed for autoradiography using Kônica X-ray film.

Enzyme-linked immunotransfer blot (EITB) was also performed according to the method of Tsang *et al.* (1983). NC papers were incubated with rabbit antisera for 1 hr and washed with PBS-Tween several times. Then incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 hr, and then washed with above buffer. NC papers were then exposed to the substrate solution (2.4 mM 4-chloro-1-naphthol in PBS containing 20% methanol with 3.5 mM H_2O_2) for 10 min.

RESULTS

1. Parasites

Pure *Toxoplasma* tachyzoite and bradyzoite preparations were required increasingly for serological, biochemical, and morphological studies. They were successfully isolated in this study with minimum damage to their nature by using physical method of Percoll density gradient centrifugation. Tachyzoites of RH strain were purified from the mouse peritoneal exudate after incubation with FMLP which activated the host leukocytes. When centrifuged over a Percoll density gradient, they were harvested at the interface of 50 and 60% Percoll solutions, and the purity of them was over 99%. Bradyzoites of tissue cyst forming Fukaya strain were also isolated by Percoll density gradient centrifugation. It demanded 2 steps of centrifugation and the cyst was harvested from

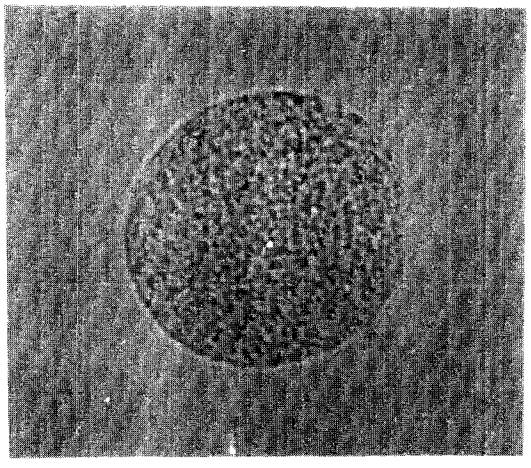

Fig. 1. Photomicrograph of a purified *T. gondii* brain cyst of Fukaya strain. Cysts were purified by Percoll density-gradient centrifugation of mouse brain infected 1 month previously.

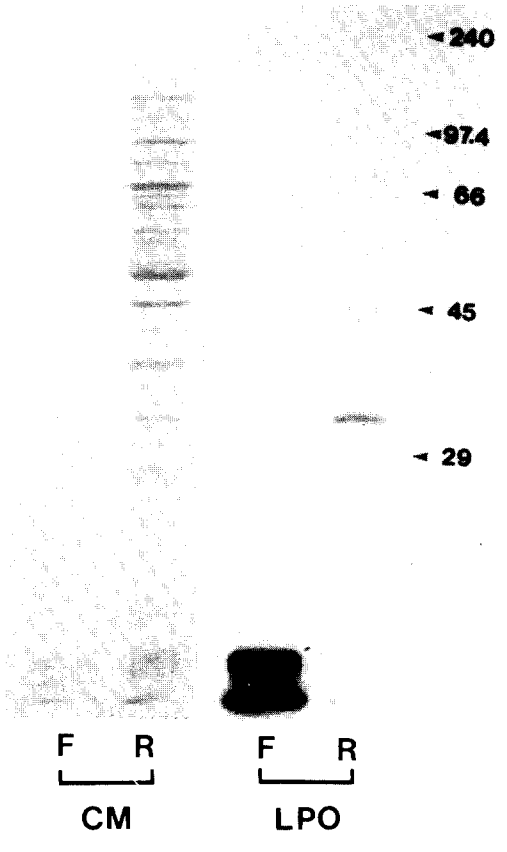

Fig. 2. Coomassie-stained pattern (CM) and lactoperoxidase(LPO)-catalyzed surface iodination pattern of bradyzoites of Fukaya strain (F) and tachyzoites of RH strain (R). Numerals stand for molecular weight ($\times10^{-3}$).

the interfaces of 40-50% and 50-60% according to the size of cyst. The purity was very high but the yield was low. The cyst isolated in this study was shown in Fig. 1.

2. Membrane Proteins

Membrane proteins were revealed by LPO-catalyzed surface iodination. There were many differences between Coomassie-stained pattern and autoradiographic pattern as shown in Fig. 2. In the Coomassie-stained lanes, bradyzoites of Fukaya strain (designated as F) showed 67 KDa, 15 KDa, 14 KDa and 12 KDa bands as major and many minor bands along the gel. The tachyzoites of RH strain (designated as R) showed many bands similar to those of general eukaryotic cells. But, in the autoradiographic pattern, it appeared the bradyzoites expressed 15 KDa and 14 KDa proteins as major and minor bands between 80 K~45 KDa proteins, and some low molecular weight proteins which were run with dye front showed active radioactivity. On the while, tachyzoites of RH strain showed 30 KDa band as major, 68 KDa, 52 KDa, 44 KDa and 32 KDa bands as minor ones.

3. Lectin Blotting

Lectins of different sugar specificities (Lis and

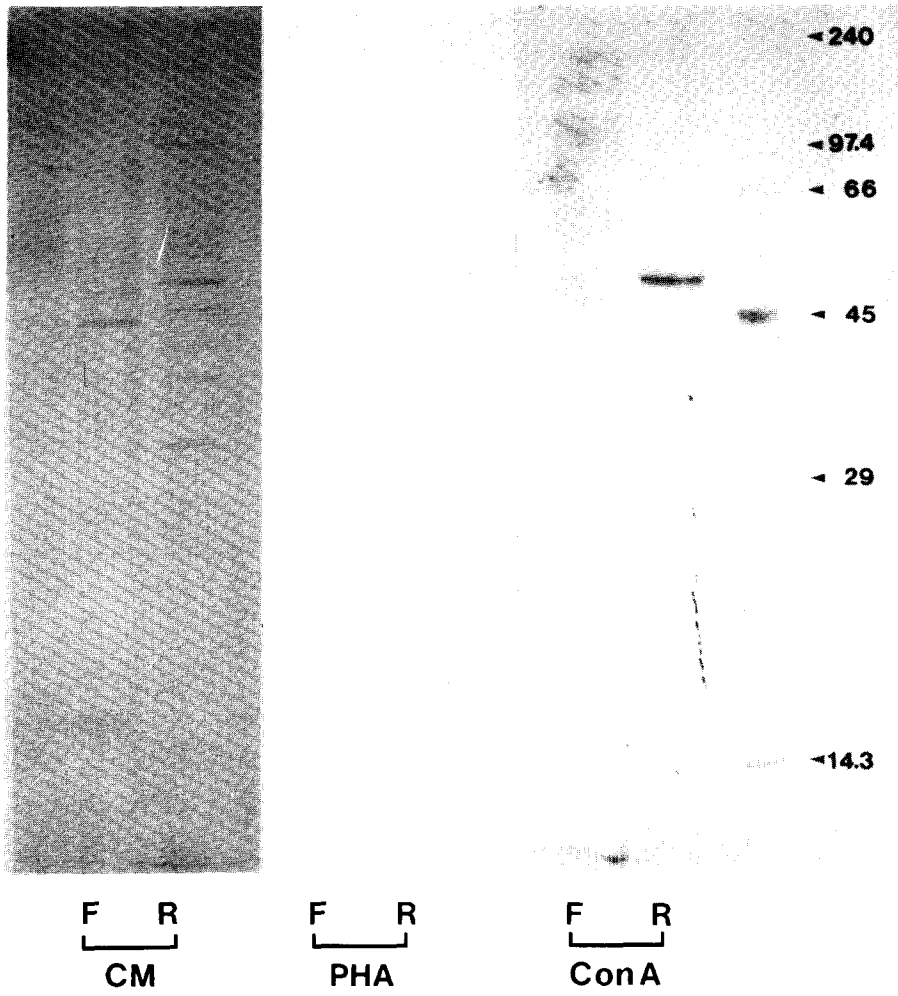

Fig. 3. Western blot analysis of bradyzoites (F) and tachyzoites (R) probed with ^{125}I-Con A (Con A) and ^{125}I-PHA (PHA). Coomassie-stained pattern was also shown as CM. Numerals stand for molecular weight ($\times 10^{-3}$).

Sharon, 1984; Ofek and Sharon, 1988) were used in this study. Con A blotting pattern revealed some bands of 200 K~50 KDa glycoproteins in bradyzoites of Fukaya strain and very different pattern of 52 KDa band as major and some bands of 33 K~20 KDa bands in tachyzoites of RH strain. But, PHA did not detect any band in bradyzoites or tachyzoites as shown in Fig. 3.

4. Enzyme-linked Immunoelectrotransfer Blot(EITB)

It appeared that both anti-Fukaya antibody and anti-RH antibody detected RH antigens and Fukaya antigens with similar cross-reactivity, but detected more RH antigens than Fukaya antigens in EITB analysis. Despite the cross-reactivity, the patterns detected showed many differences as shown in Fig. 4. Anti-Fukaya antibody detected 68 KDa, 50 KDa, 32 KDa and 15 KDa bands as major in Fukaya antigens and several bands of 100 K~32 KDa but not 15 KDa band in RH antigens. Anti-RH antibody reacted with 68 KDa and 28 KDa bands as major and 65 K~45 KDa bands as minor in Fukaya antigens, while 68 KDa, 52 KDa, 40 KDa and 30 KDa as major and several bands in RH antigens. Therefore, 15 KDa protein of Fukaya antigens and 52 KDa, 30 KDa, and 25 KDa proteins appeared to be stage-specific antigenic proteins.

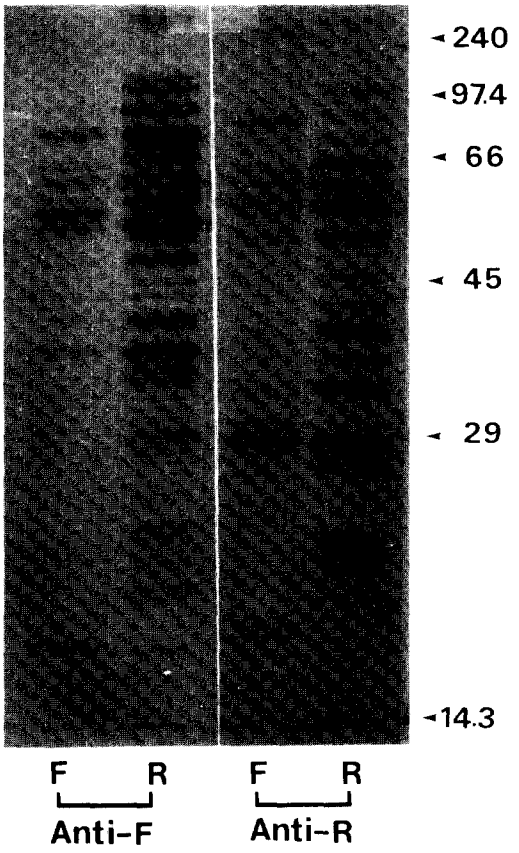

Fig. 4. EITB patterns of bradyzoites (F) and tachyzoites (R). Reactions with anti-Fukaya antibody were designated as Anti-F and those with anti-RH antibody were described as Anti-R. Numerals stand for molecular weight ($\times 10^{-3}$).

DISCUSSION

Many methods have been proposed for the purification of *Toxoplasma gondii* zoites from peritoneal exudates or brain tissues of infected mice (Tryon and Weidner, 1978; Grimwood *et al.*, 1979; Pettersen, 1979; Cornelissen *et al.*, 1981; Dempster, 1984). For the biochemical purposes chemicals or enzymes could not be used as possible, there were no such materials as described above which could affect the nature of *Toxoplasma* proteins but FMLP in the case of purification of RH tachyzoites from peritoneal exudates in this study. Therefore, the physical procedures adopted in this study might be guaranteed for the natural properties of *Toxoplasma* proteins. And, in addition to the high purity of tachyzoites or bradyzoites, the yield was also appreciable to some degree.

In spite of many reports concerned with *Toxoplasma* membrane proteins, there was no report which dealt with the relationship between antigenicity and glycoconjugate. Glycoproteins of other species were studied extensively (Jaffe and McMahon-Pratt, 1988; Rodriguez *et al.*, 1988; Balber and Frommel, 1988; Petri *et al.*, 1987) in order to identify the species, to define the recognition process in host-parasite relationship, and to isolate the fucose-mannose receptor (FMR) which is focused as a new receptor additional to Fc receptor or C_3 receptor.

In this study, lectin-binding glycoproteins of tachyzoites of RH strain and bradyzoites of Fukaya strain were defined using mannose-specific Con A and galactose-specific lectin(phytohemagglutinin) and compared to antigenicity and membrane locality. In the autoradiogram obtained using LPO-catalyzed surface iodination of RH tachyzoites showed membrane proteins of 68 KDa, 44 KDa, and 32 KDa as minor and 30 KDa protein as major ones. These results agreed with those of previous reports by Handman *et al.* (1980), Johnson *et* al. (1983), Araujo *et al.* (1984), and Kasper *et al.* (1984 & 1985). And, in the autoradiogram bradyzoites showed the prevalence of low molecular weight proteins of 15 KDa and 14 KDa on the surface of that organism. But, when compared with those patterns of lectin blotting, there was a dogmatic phenomenon. The patterns of glycoproteins of both tachyzoites and bradyzoites appeared different in molecular weight distribution. In bradyzoites, high to middle molecular weight proteins of 200 K~ 50 KDa appeared to be glycoprotein and in tachyzoites, 52 KDa band and low molecular weight below 33 KDa represented glycoproteins. There was little knowledge on the function of glycoproteins of *Toxoplasma*, these biased distribution of glycoproteins shieled by major surface proteins suggested the strategies of this organism to adapt to parasitism.

Furthermore, when comparing above results with enzyme-linked immunotransfer blot (EITB) pattern, both anti-Fukaya antibody and anti-RH antibody detected crossly Fukaya antigens and RH antigens. This result presented the rationale of use of RH antigen to diagnose toxoplasmosis (Choi *et al.*, 1987). But, it appeared that stage-specific antigenicity was present in *Toxoplasma* such as 15 KDa protein in Fukaya strain and 52 KDa, 30 KDa, and 25 KDa proteins in RH strain. Therefore, it might be focused on the more precise research and diagnosis. In the case of tachyzoites, 52 KDa surface glycoprotein (gp52) must be focused additionally to the major surface protein p30 of tachyzoites (Santoro *et al.*, 1985; Kasper *et al.*, 1986).

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=국문초록=

*Toxoplasma gondii*의 세포막 단백 성분과 그 항원성

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*Toxoplasma gondii*의 강독주인 RH주와 조직내 cyst 형성주인 Fukaya주의 세포막 단백 성분을 SDS 존재하에서 전기영동하여 분석하였다. 먼저 RH tachyzoite와 Fukaya의 cyst를 각각 마우스의 복강액과 뇌조직으로부터 분리하였는데, 불연속 Percoll density-gradient에서 원심분리하여 tachyzoite는 50 %와 60 % Percoll용액 경계면에서, cyst는 40 %와 50 %의 경계면 및 50 %와 60 % 경계면에서 얻었으며, cyst는 저장액으로 처리하여 bradyzoite를 얻었다.

Lactoperoxidase를 촉매로 세포막에 방사성 요오드를 표지시킨 후 자가방사표지그림을 얻었을 때, bradyzoite는 15 KDa와 14 KDa의 분자량을 가진 단백질이 주요 단백질로 나타났으며, tachyzoite에서는 30 KDa 단백질이 주요 단백질로 나타났다. 또, 당단백질의 존재를 파악하기 위해서 lectin blotting을 시행하였는데, concanavalin A는 bradyzoite에서 200 K~50 KDa의 여러 단백질을, 그리고 tachyzoite에서는 52 KDa 단백질을 주로 하는 33 K~20 KDa 단백질을 검출하였으며, phytohemagglutinin은 두 유형에서 아무런 단백질도 검출하지 못하였다. 한편, 이들을 효소면역이적법으로 항 Fukaya항체와 항 RH항체로 반응시켰을 때, 많은 교차 반응을 보였으나, bradyzoite에서는 15 KDa 단백질이, 그리고 tachyzoite에서는 52 KDa, 30 KDa 및 25 KDa 단백질이 각각 유형특이 항원 단백으로 나타났다.

위의 결과들로, bradyzoite에서는 15 KDa 단백질이 당단백질은 아니지만 특이 항원성을 갖는 주요 세포막 단백으로 나타났으며, tachyzoite에서는 지금까지 주요 세포막 단백으로 알려진 p30 외에 당단백질이며 특이 항원성을 갖는 세포막 단백으로 52 KDa 단백(gp52)을 확인할 수 있었다.