Immunoglobulin M and G Antibodies in Mice in Response to *Toxoplasma gondii* (S-273) Infection and Their Antigen Recognition Patterns in Western Blotting on Various Post-Infection Days

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Abstract

Immunoglobulin M and G antibody responses in mice experimentally infected with *Toxoplasma gondii* (S-273) and the reaction patterns with *T. gondii* (RH) tachyzoite antigens were studied on various post-infection days (PIDs) (2nd to 36th PIDs) using a commercially available IgM and IgG enzyme linked immunosorbent assay (ELISA) test systems and Western blotting (WB) technique. IgM antibody in ELISA test appeared to be positive on 12th PID (absorbance 0.764) and reached its peak level on 16th PID (absorbance 1.338) showing a slow decline thereafter with an absorbance of 0.800 even on 36th PID. Positivity of IgM was confirmed by WB except for 36th PID. IgG appeared on 16th PID with an absorbance of 0.248 and showed a steady increasing tendency even on 36th PID (absorbance 1.747). However, IgG positivity on WB was observed only on 29th PID and afterwards. On Western blots, both IgM and IgG showed interesting antigen recognition patterns on various PIDs. At the most IgM recognised seven antigens of 14kDa to 53kDa while IgG recognised eight antigens of 17kDa to 53kDa. Major antigens recognised by IgM were of 53kDa and 21kDa while the major band recognised by IgG was of 19kDa. The major bands, however, showed variability in their consistency during various PIDs. All the antigens recognised by IgM and IgG were not identical.

Introduction

Although very sensitive and specific diagnostic test such as polymerase chain reaction (PCR) using various primers has been developed recently1, serological tests remain the mainstay in the diagnosis of *Toxoplasma gondii* infections in both man and animals. This is because of its ease, cost-effectiveness and more importantly, because of its ability to discriminate between acute and chronic stages of the infections2. Recently, besides IgM, *Toxoplasma* specific IgA3,4 and IgE5...
detection has also been suggested to be more helpful in the diagnosis of acute, reactivated and congenital *T. gondii* infection. It is, however, not true for AIDS patients because of failure in antibody production or significant delay in its production\(^2\). However, lymphocytes from AIDS patients\(^6\) and primarily infected immunocompetent patients\(^7\) have been reported to synthesize *Toxoplasma* specific antibodies *in vitro* and are thought to be useful biological markers for the diagnosis of toxoplasmic encephalitis. On the other hand, various different types of antigen recognition patterns have been shown by other workers previously by Western blotting (WB) during different stages of the infection\(^8\)-\(^10\) and in congenitally infected newborns and their mothers\(^11\). We therefore became interested to study the IgM and IgG antibody responses in mice experimentally infected with *T. gondii* (S-273) through oral route and to determine their antigen recognition patterns on Western blots on various post-infection days (PIDs) in order to see whether the antigen recognition patterns by IgM and IgG differ even during acute infection.

**Materials and Methods**

A total of five ICR albino mice (Nippon CLEA Company, Japan) were infected orally with weakly pathogenic strain of *T. gondii* (S-273) tissue cyst. Mouse brain tissue containing approximately 20 cysts/4 mg of tissue was gently homogenized in 5 ml of minimum essential medium (MEM) and 0.1 ml of brain tissue homogenate was fed to each mouse used in this study. From each mouse about 70 µl of blood sample was collected from the tail vein cut using capillary tubes on 2th, 4th, 6th, 8th, 12 th, 16th, 20th, 24th, 29th and 36th post-infections days and were pooled immediately into microtainer serum separator tube (Becton Dicknson, USA). Serum samples separated were then stored at \(-20^\circ\)C till the tests were performed. *Toxoplasma* antibodies of IgM and IgG classes were measured by ELISA using commercially available test reagents (Labozyme Toxoplasma IgM-EIA and Labozyme Toxoplasma IgG-EIA; Labsystems, Finland) with modification. We used peroxidase-rabbit anti-mouse IgM and peroxidase-goat anti-mouse IgG (Zymed Laboratories, Inc., USA) conjugates. Tests were performed manually and the results were read in ELISA reader (Sanko Junyaku Co., Ltd., Japan: Model ER-8000). Serum samples were pre-treated as described by the manufacturer prior to performing the IgM-ELISA test as described for human sera. The specificity of ELISA was checked by inhibition ELISA using *T. gondii* (RH) whole cell and sonicated antigens absorbed sera.

All the serum samples were then subjected to WB using *Toxoplasma* antigen blotted polyvinylidine difluoride (PVDF) microporous membrane (Milipore Corporation, USA). In brief, a sonicated (60 cycles for 30 seconds) antigen was prepared from *T. gondii* (RH) tachyzoites harvested from ICR albino mice which had been intraperitoneally infected three days earlier. The antigen was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 25 mA constant current) as described by Laemmlli\(^12\) along with the following pre-stained molecular weight standards: phosphorylase b (94kDa), albumin (76kDa), ovalbumin (43kDa), trypsin inhibitor (20kDa) and alpha-lactalbumin (14kDa) (Pharmacia AB, Sweden). Proteins separated by SDS-PAGE were transferred to PVDF membrane using an electroblotter (JNSEB, Jooko Corporation, Japan) at 100 mA for 50 minutes. Blots were first soaked in 5.0% skim milk (Difco Laboratories, USA) in Tris-tween-20 buffered saline (blocking buffer) for 1 hr at room temperature and then dried using blotting paper. Western blotting was performed as described previously\(^9\). The PVDF membrane was cut into strips which were incubated overnight with the test sera of various PIDs diluted 1:100 in blocking buffer. After washing three times in washing buffer (Tris-NaCl-Tween-20), the blots were incubated at room temperature with horseradish peroxidase labelled rabbit anti-mouse IgM and goat anti-mouse IgG antibodies (Zymed Laboratories, Inc., USA) separately at a dilution of 1:1000. After another wash, the blots were soaked in 0.05% 4-chloro-m1-naphthol and 0.1% hydrogen peroxide in Tris-buffered saline.
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Fig. 1 Toxoplasma antibody level on various post-infection days in mice infected with T. gondii (S273) as measured by ELISA (IgM-ELISA and IgG-ELISA) methods.

Fig. 2 Diagramatic representation of T. gondii antigen recognition patterns of mice Immunoglobulin M and G produced during various post-infection days (PIDs).

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<th>IgM Antibody</th>
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(TBS) for 10 min. Colour development was stopped by washing with the washing buffer.

Results

Toxoplasma antibodies were detectable by ELISA on 12th PID and onwards (Fig. 1). The antibody that appeared on 12th PID (absorbance 0.764) and onwards was of IgM type which reached its peak level on 16th PID with an absorbance of 1.338 and began to decline slowly thereafter. The level of IgM, however, persisted at significantly high level (absorbance 0.8) until 36th PID. IgM positivity on ELISA test was confirmed by WB except for 36th PID (Fig. 2). IgG appeared on 16th PID with an absorbance of 0.248 and showed a steady increase even until 36th PID with an absorbance of 1.747 (Fig. 1). However, IgG positivity in WB was observed only on 29th and 36th PIDs (Fig. 2). On Western blots, both IgM and IgG showed interesting antigen recognition patterns on various PIDs (Fig. 2). IgM on 12th PID recognised antigens of molecular weights 53kDa, 50kDa and 21kDa of which 53kDa and 21kDa bands were bigger and intenser. The same was true for 16th PID, but with the appearance of additional bands of 30kDa, 20kDa, 19kDa and 14kDa. The 20kDa band was seen only on 16th PID. The 50kDa, 30kDa and 14kDa bands disappeared on 24th PID and thereafter, while, the 53kDa, 21kDa and 14kDa bands persisted until 29th PID. It was interesting to note that the high intensity bands of 53kDa and 21kDa showed a thinning tendency on 20th PID and thereafter along with the increase of IgG. IgM recognised seven antigens having mol. wts. of 14kDa
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to 53kDa. IgG showed its positivity in WB only on 29th PID with the recognition of bands of 30kDa, 28kDa, 23kDa, 21.5kDa and 19kDa. On 36th PID, three additional bands of 53kDa, 50kDa and 17kDa appeared, although 19kDa band was the major one. Taken together, antigen recognition patterns of mouse Toxoplasma IgM and IgG were of different types on various PIDs. The very low level of IgM observed on 8th PID, however, was not confirmed in WB and was considered to be negative.

A significantly reduced ELISA values (absorbance of 0.145 and less than 0.145) were observed in inhibition ELISA on various PIDs.

**Discussion**

In the present study, we studied the IgM and IgG responses in ICR mice experimentally infected with *T. gondii* (S-273) cysts obtained from previously infected mice. The main purpose of this study was to see the IgM and IgG responses and their antigen recognition patterns during various PIDs. In this study, IgM and IgG antibodies were detectable by ELISA methods on 12th and 16th PIDs respectively. Handman & Remington\(^{13}\), however, have reported IgM and IgG antibody positivity in mice as early as second and fifth PIDs, respectively. This could be due to the differences in the strain and stage of the parasite and the route of infection. (They inoculated tachyzoite form of C37 strain subcutaneously.) The IgM antibody positive by EIISA on 12th PID and thereafter was confirmed by WB. The IgM antibody attained its peak level on 16th PID and then showed a slow decline, and could be detected even on 36th PID with an absorbance of 0.8. This finding supports that Toxoplasma IgM antibody can persist for a long period (several months) as reported earlier by various workers\(^{3,4,5,11,13}\) making IgM detection alone not much useful in the diagnosis of acute infection. However, failure to observe any band on Western blots could be due to competition between large amounts of IgG and relatively little amount of IgM in the serum sample. Our finding showed domination of IgM over IgG even when the IgM level was either equal or less than the IgG level as shown by ELISA on 24th and 29th PIDs. This appears to be because of its more antigen binding sites compared to those of IgG.

On the other hand, though *Toxoplasma* IgG in ELISA was positive on 16th PID and thereafter, no bands in WB could be seen until 29th PID. This again appears to be due to competition between IgM and IgG for the antigen binding sites and domination of IgM over IgG as mentioned earlier. The IgG was still showing a tendency to increase until 36th PID. We could not see the peak level attained by IgG followed by a declining tendency as we terminated our experiment on 36th PID.

Besides the competitive antigen recognition patterns between IgM and IgG, WB analysis also revealed different interesting antigen recognition patterns shown by both IgM and IgG on various PIDs. IgM, for the first time on 12th PID, recognised the antigens of 53kDa, 50kDa and 21kDa, out of which 53kDa and 21kDa bands were remarkably prominent. Three days later, on 16th PID, four additional bands of 30kDa, 20kDa, 19kDa and 14kDa were observed. Same was true for 20th PID also, except for the disappearance of the 20kDa band. On 24th and 29th PIDs, the 50kDa, 30kDa and 19kDa bands also disappeared. Moreover, the more prominent bands of 53kDa and 50kDa also showed a thinning tendency on 20th PID and thereafter, with the increase of serum IgG level. Thinning of bands with time has been observed in human sera obtained from a case of laboratory acquired toxoplasmosis also which persisted until 9th week\(^{11}\). The IgM recognised at least three to seven antigens depending on the duration of infection. A similar striking pattern of bands was observed with IgG on 29th and 36th PIDs. On 29th PID, IgG recognised antigens of 30kDa, 28kDa, 23kDa, 21.5kDa and 19 kDa. In addition to these, three more bands of 53kDa, 50kDa and 17kDa were seen on 36th PID. Interestingly, the 19kDa band became more prominent on 36th PID. Our present study has clearly shown that the antigen recognition patterns differ not only among the IgGs produced during acute and chronic *Toxoplasma* infection as reported by Suzuki et al.\(^{8}\), but also differ in the IgMs and IgGs.
produced during different stages of the primary infection. None of the bands seen before 29th PID appears to be due to competition between IgM and IgG, and IgM domination. We, however, could not examine this effect of IgM by treating the sera with 2-mercaptoethanol because of insufficient serum sample. The difference in the antigen recognition patterns between IgM and IgG could be due to their preference primarily to polysaccharide and protein antigens, respectively, as reported earlier by Mineo et al.14. Determination of the factor responsible for the appearance of different band patterns on various PIDs requires further studies.

We checked the specificity of ELISA used in this study inhibition ELISA with the use of *T. gondii* whole cell and sonicated antigens absorbed sera and found to be highly specific (an absorbance of 0.145 even on PID 36).

Our present WB analysis of mice serum were different from those reported by other workers in human studies9,11,15,16. In humans too, variability in the antigen recognition patterns in the human sera of acute and chronic stages of the infection9,19, congenitally infected newborn and their mothers10 and different stages of laboratory acquired infection11 has been noticed. Our present findings, however, suggest that the antigen recognition patterns of both IgM and IgG do differ even during different stages of primary/acute infection at least in case of mice. Similar findings in human was reported by Partanen et al.11, but the bands in their study persisted for 9 weeks. Moreover, the intensity of the same molecular band also differs with the stage of infection probably due to competition between IgM and IgG. In this study, however, we could not see the prominence of the 30kDa antigen recognised previously by other workers as P30 antigen19,17. This could be due to the difference in antigen preparation method. Further studies using antigens prepared from different strains of *Toxoplasma* (tachyzoite) by various methods are proposed herewith.

**References**


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要 旨

実験的にトキソプラズマ（S-273株）を感染させたマウスの免疫グロブリンMおよびGの変動と、感染経過日に見られるウエスタンプロットの対応抗原パターン

(平成6年11月20日)