Antibody Responses to *Toxoplasma gondii* in Sera, Intestinal Secretions, and Milk from Orally Infected Mice and Characterization of Target Antigens

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*Toxoplasma gondii*-specific antibody responses in serum, intestinal secretions, and milk were identified with an enzyme-linked immunosorbent assay following a single oral infection of mice with strain 76K cysts of *T. gondii*. Immunoglobulin A (IgA) production began during week 2 of infection in serum and milk and during week 3 of infection in intestinal secretions and persisted in all three throughout the experiment (17 weeks). IgG but not IgM antibodies were detected in intestinal secretions later in the infection. Serum and milk IgG and IgM production began at the same time after infection as did the IgA response. In Western blotting (immunoblotting), intestinal IgA antibodies were shown to react with antigens comigrating with the *T. gondii* proteins p22, p23, p30, and p43, the 28-kilodalton antigen, and the 55- and 60-kilodalton rhoptry proteins, as recognized by specific monoclonal antibodies. Milk IgA antibodies reacted with antigens comigrating with p30 and p43. Most of the antigens recognized by IgA antibodies were also detected by IgG antibodies. IgA antibodies from all three biological samples detected the same major *T. gondii* antigens; thus, there was apparently no specific antibody production unique to one locality.

Toxoplasmosis, a coccidian infection, is caused by the obligate intracellular parasite *Toxoplasma gondii*. Although generally benign for healthy people, infection can result in stillbirth, blindness, mental retardation, and occasionally the death of congenitally infected infants (12). More recently, this disease has been observed in immunocompromised patients, particularly those with acquired immunodeficiency syndrome but also those with neoplastic disease and bone marrow or heart transplant recipients (12). In veterinary medicine, cattle abortion and neonatal loss due to toxoplasmosis are of great economic importance in many parts of the world (19). Infection with *T. gondii* most commonly occurs via the oral route (10, 13) and induces specific secretory immunoglobulin A (IgA) synthesis in the gut (23). Secretory IgA antibodies are considered to be protective against intestinal coccidiosis through the inhibition of cell penetration by the parasite and of its subsequent intracellular development (6, 7). Important insights into the mechanisms that confer resistance to oral or congenital infection may be gained by studies of toxoplasma-specific IgA antibodies arising after infection. With this perspective in mind, using an enzyme-linked immunosorbent assay (ELISA), we monitored the kinetics of the IgA antibody response in serum, intestinal secretions, and milk from orally infected mice and made a comparison with the IgG and IgM antibody responses. Furthermore, we used Western blotting (immunoblotting) to detect the *T. gondii* antigens recognized by these antibodies.

**MATERIALS AND METHODS**

**Parasites.** Two strains of *T. gondii*, RH and 76K, were used in this study. Strain 76K is a low-virulence strain isolated by Laugier and Quilici from a guinea pig (18). When inoculated into a mouse, this strain regularly causes latent toxoplasmosis, with the production of a large quantity of cysts. It was used solely for the peroral infection of mice and was passed every 2 months by intragastric gavage of cysts collected from the brains of infected animals. Otherwise, RH strain tachyzoites were used throughout and were routinely maintained by serial passage in the peritoneal cavities of OF1 mice.

**Mice.** Six- to 8-week-old OF1 female mice (IFAA Credo) were used throughout. They were raised in an air-conditioned building (21°C and 60% relative humidity) in a room with controlled daily lighting and had free access to sterile food and water. Day 1 of gestation, for those mice which were mated, was taken as the time at which vaginal plugs were first detected.

**MAbs.** The production of the anti-*T. gondii* monoclonal antibodies (MAbs) has been described elsewhere (3, 30). The following MAbs were used: 3F12 (recognizing an antigen of 4.5 kilodaltons [kDa]), 3G11 (anti-p22), 2E12 (anti-p23), 1F5 (recognizing an antigen of 28 kDa), 1E5 (anti-p30), 1F12 (anti-p43), and 4A7 (anti-55- and 60-kDa rhoptry proteins).

**Peroral infection with *T. gondii*.** Brain tissue containing strain 76K cysts was triturated in saline, and the suspension was adjusted to contain 40 cysts in each 0.5-ml dose. The cysts were administered intragastrically to nonpregnant and pregnant mice by gavage with a specially designed intubation needle (day 0 of infection). In the case of pregnant mice, infection was performed 5 days before parturition. Six to 10 mice were used for each experiment.

**Sample collection.** Blood and intestinal secretions were collected on day 0 before infection and thereafter once per week until day 122. Milk samples were collected from lactating mice on days 7, 10, 14, 17, 21, 24, and 27 following infection. Intestinal secretions were recovered and processed by the lavage technique described by Elson et al. (11), except that the dose of pilocarpine was 0.2 mg per mouse.
Lactating mice were milked by the technique of Nashar et al. (25). Blood samples were collected by retro-orbital puncture. All three biological fluids samples were stored frozen at -20°C until assay.

ELISA. A modified version of the ELISA described by Naot and Remington (24) was used to detect anti- *T. gondii* IgA, IgG, and IgM antibodies in the three biological fluids. Flat-bottomed wells of microdilution plates (Nunc) were coated with 50 μl of *T. gondii* tachyzoites (40 × 10⁶ ml⁻¹) in 150 mM pH 7.4 phosphate-buffered saline (PBS). After centrifugation at 200 × g and 4°C for 5 min, 20 μl of 0.5% glutaraldehyde in cold PBS was added to each well and left for 8 min at room temperature. The plates were washed twice in PBS and postcoated with 4% bovine serum albumin (Sigma Chemical Co.) in PBS for 1 h at 37°C. Threefold serial dilutions of the samples were made in PBS, with an initial 1/15 dilution for serum and milk samples and with initially undiluted intestinal secretions. After three washes in PBS containing 0.05% Tween 20, 100 μl of the samples (two replicates for each dilution) was added to each well and the plates were incubated for 1 h at 37°C. After three washes in PBS-0.05% Tween 20, 100 μl of goat anti-mouse immunoglobulin-alkaline phosphatase conjugate was added to each well. The conjugate was anti-IgA, -IgG, or IgG (Sigma), as appropriate, and was used at a 1:1,000 dilution in 4% bovine serum albumin-PBS. The plates were incubated for 2 h at 37°C, followed by three washes in PBS-0.05% Tween 20. One-hundred microliters of a 1-mg ml⁻¹ 4-nitrophenylphosphate disodium (Sigma) solution in 1 M pH 9.8 diethanolamine was added to each well. After 20 min of incubation for serum and milk assays and 60 min of incubation for intestinal secretions, the A₄₅₀ was determined with an automatic ELISA reader (Multiskan MCC340; Flow Laboratories, Inc.).

**SDS-polyacrylamide gel electrophoresis and Western blotting.** Before electrophoresis, purified tachyzoites were heated with sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.005% bromophenol blue in 62.5 mM pH 6.8 Tris) at 100°C for 4 min. The lysates (25 × 10⁶ tachyzoites 1-cm-wide slot⁻¹) were separated by SDS-polyacrylamide gel electrophoresis with an apparatus from BioRad Laboratories and 15% (wt/vol) acrylamide separating gels under nonreducing conditions and then electrophoretically transferred to nitrocellulose sheets (0.45 μm; Schleicher & Schuell, Inc.) with a semidyed fast blot system (Biometra). The buffer consisted of 48 mM Tris, 39 mM glycine, 0.0375% (wt/vol) SDS, and 20% methanol (pH 8.3). A constant current of 0.8 mA cm⁻² was applied for 1 h at room temperature. The nitrocellulose sheets were saturated for 1 h with 5% nonfat powdered milk in TNT buffer (140 mM NaCl, 0.05% [vol/vol] Tween 20 in 15 mM pH 8.8 Tris). Strips were cut and incubated with different samples (serum and milk diluted 1/100 in TNT, intestinal secretions diluted 1/3 in TNT, MAbs diluted 1/200 in TNT) for 1 h. After three washes in 5% nonfat powdered milk-TNT, the strips were incubated for 1 h in alkaline phosphatase-anti-mouse IgA, IgM, or IgG conjugate (Sigma) diluted 1/1,000 in 5% nonfat powdered milk-TNT. Strips were washed three times in TNT and finally in R buffer (100 mM Tris hydrochloride, 100 mM NaCl, 5 mM MgCl₂ [pH 9.5]). Stock solutions for the alkaline phosphatase reaction were prepared as follows: 50 mg of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide (Promega Biotec) ml⁻¹ and 50 mg of Blue TetrAzolium in 70% dimethylformamide (Promega) ml⁻¹. The reaction solution (16.5 μl of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide, 33 μl of Nitro Blue TetrAzolium in 70% dimethylformamide, and 5 ml of R buffer) was added to the washed nitrocellulose strips. The development of the reaction was stopped with 20 mM pH 8 Tris–5 mM EDTA buffer. Molecular weight standards (MW-SDS-70L; Sigma) used for calibration were stained nonspecifically with 0.2% Ponceau Red solution (Ponceau S; Serva) in 3% trichloroacetic acid.

**Datum analysis.** Data were expressed as the mean value plus or minus the standard deviation. The paired Student’s t test was used to determine the significance of differences between data before infection and during the course of infection (IgA, IgG, and IgM titers). Results for which P was ≤0.05 were defined as statistically significant.

**RESULTS**

**Time course of intestinal, milk, and serum anti-*T. gondii* antibody production (IgA, IgG, and IgM) after oral infection.** Serum IgA to *T. gondii* antigens was first observed on day 14 (P < 0.01), peaked on day 42 (P < 0.001), and persisted throughout the experiment (day 122) (Fig. 1). Similarly, serum IgG and IgM were detected from day 14 onwards (P < 0.001). However, the serum anti- *T. gondii* IgG titer reached a plateau on day 28 (P < 0.001) and did not change significantly throughout the rest of the study, whereas the serum anti- *T. gondii* IgM titer peaked on day 14 (P < 0.001) and then decreased progressively (Fig. 1).

In intestinal secretions, IgA was first observed on day 21 (P < 0.05); the response peaked on day 49 (P < 0.005) and persisted, at a low titer, to the end of experiment (day 122) (Fig. 2). No IgM was detected in intestinal secretions. In contrast, a significant (P < 0.05) increase in the intestinal anti-*T. gondii* IgG titer was detectable, but only from day 28 of infection onwards (Fig. 2).

A milk IgA response began between days 10 and 14 of infection (P < 0.05) and persisted throughout the experiment (day 27) (Fig. 3). A significant increase in the milk IgG and IgM responses was detected at the same time (P < 0.01 and P < 0.05, respectively). However, the increase in the IgG and IgM titers observed between days 7 and 10 postinfection was not significant. The IgM titer peaked on day 14 and thereafter slowly declined, whereas the IgG antibody response remained on a plateau until the end of the study (Fig. 3).
Toxoplasma antigens recognized by serum IgA, IgG, and IgM antibodies during the course of infection. Serum IgA antibodies recognized 12 major antigens with apparent molecular masses of 4.5, 21, 24 (faint band), 27, 28 (faint band), 30, 33, 38, 40 (faint band), 50, 55, and 60 (faint band) kDa. The time course of detection was as follows: 27- and 30-kDa bands (faint) on day 7, 38-kDa band on day 14, 4.5-, 21-, faint 24-, 40-, and 50-kDa bands on day 35, 28-kDa band (faint) on day 49, and 33-, 55-, and 60-kDa bands (faint) on day 70. After day 105 until the end of the experiment, only the 27-, 30-, 38-, 40-, 50-, 55-, and 60-kDa bands persisted. Maximum detection, as shown by the intensity of the bands, occurred between days 35 and 70.

The major 30-kDa antigen was also detected by IgM antibodies on day 7, with maximum band density on day 14. The 21-, 24-, faint 27-, 33-, 38-, 40-, and 50-kDa bands were first observed on day 14. All the bands then gradually decreased in intensity until the end of the experiment or ceased to be detected. Furthermore, a wide, 4.5-kDa band was recognized by IgM antibodies from day 42 to the end of the study.

The serum anti- *T. gondii* IgG antibodies revealed a complex pattern of antigenic components over a wide molecular mass range of 16 to 70 kDa. The major bands detected by serum IgA antibodies were also recognized by serum IgG antibodies, but the bands were more intense and the kinetics were different. Antigens of 21, 24, 27, 28, 30, 38, 40, 50, 55, and 60 kDa were revealed from day 14 onwards, and antigens of 4.5 and 33 (faint band) kDa were revealed from day 35 onwards.

Finally, eight major bands (4.5, 21, 24, 28, 30, 38, 55, and 60 kDa) recognized by serum anti-*T. gondii* antibodies showed a migration pattern similar to that of major toxoplasma antigens (4.5-kDa antigen, p22, 28-kDa antigen, p30, p43, and 55- and 60-kDa rhoptry antigens, respectively), as recognized by MAbs (Fig. 4).

**Toxoplasma antigens recognized by intestinal IgA and IgG antibodies during the course of infection.** Western blotting revealed reactions between intestinal anti-*T. gondii* IgA antibodies and nine major bands with apparent molecular masses of 21, 24 (faint band), 28, 30, 38, 40 (faint band), 50, 55, and 60 kDa. The 30-, 55-, and 60-kDa antigens were first detected on day 14. The 21-, 24-, and 38-kDa antigens were only recognized on day 35, and the 28-, 40-, and 50-kDa bands were only recognized on day 49. From day 70 to the end of the study, the only antigens which continued to be detected were the 21-, 30-, 55-, and 60-kDa bands. Maximum detection, as shown by the intensity of the bands, occurred on day 49.

The intestinal IgG antibody response was directed against the same 21-, 24-, 30-, 38-, 55-, and 60-kDa antigens recognized by the intestinal IgA antibodies. The time course of detection was as follows: 55- and 60-kDa bands on day 14, 30- and 38-kDa bands on day 21, and 21- and 24-kDa bands on day 35. All the bands detected by IgG antibodies persisted with variable intensity during the course of infection, with maximum detection on day 70.

Finally, the 21-, 24-, 28-, 30-, 38-, 55-, and 60-kDa antigens recognized by intestinal anti-*T. gondii* antibodies migrated in a fashion similar to that of major toxoplasma antigens (p22, p23, 28-kDa antigen, p30, p43, and 55- and 60-kDa rhoptry proteins, respectively), as detected by specific MAbs (Fig. 5).

**Toxoplasma antigens recognized by milk IgA, IgG, and IgM antibodies during the course of infection.** Three antigens with apparent molecular masses of 30, 38, and 40 kDa were detected by milk anti-*T. gondii* IgA antibodies with a Western blot technique. The 30-kDa band was first observed on day 10 of infection, whereas the 38- and 40-kDa bands were only apparent on days 14 and 24, respectively. The detection of these three antigens persisted until the end of lactation.

Similarly, the 30- and 38-kDa bands were recognized by milk IgM antibodies, with detection first occurring on day 10, when the bands were most intense. The intensity of the 30-kDa band gradually decreased until the end of lactation, while the 38-kDa band ceased to be detected after day 17 of infection.

The milk IgG migration pattern was more complex. Ten major bands with apparent molecular masses of 21, 24, 28, 30, 38, 40, 50, 55, 60, and >66 kDa were detected by milk anti-*T. gondii* IgG antibodies. The time course of detection was as follows: 30- and 38-kDa bands on day 10, 40-, 50-, 55-, 60-, and >66-kDa bands on day 14, 21- and 24-kDa bands on day 17, and 28-kDa band on day 21. The 30-, 38-, and 40-kDa antigens were identical to those recognized by milk IgA antibodies. The detection of all the antigens persisted until the end of lactation.
Seven antigens recognized by milk anti-*T. gondii* antibodies (21-, 24-, 28-, 30-, 38-, 55-, and 60-kDa bands) showed a migration pattern similar to that of major toxoplasma antigens (p22, p23, 28-kDa antigen, p30, p43, and 55- and 60-kDa rhoptry proteins, respectively), as detected by specific MAbs (Fig. 6).

**DISCUSSION**

Previous reports have demonstrated intestinal IgA antibodies (1, 7, 16, 17, 26, 28) and milk IgA antibodies (4, 5, 15, 21, 33) following bacterial, viral, or parasitic infection. In some cases, the presence of pathogen-specific IgA antibodies has been associated with protection against infection by the pathogen (2, 5, 14, 17, 26, 28). McLeod and Mack (23) first demonstrated the production of intestinal IgA antibodies to *T. gondii* and later suggested that IgA may play a protective role against toxoplasmosis (22).

Following oral infection of mice with strain 76K toxoplasma cysts, we were able to detect serum, intestinal, and milk IgA antibodies to *T. gondii* and to specify the kinetics of the response by using an ELISA. The IgA antibody response began earlier in serum and milk (week 2 after infection) than in intestinal secretions (week 3). Nevertheless, at the intestinal level, the IgA antibody response was the first humoral response, whereas in milk and serum, IgA, IgG, and IgM production all commenced at the same time after infection. Initially, the IgA and IgM antibody titers in serum and intestinal secretions rose in parallel, as described by others for serum antibodies (29), but the IgM antibody titers peaked earlier than the IgA antibody titers. The time course of IgA production apparently began slightly earlier in Western blotting than in the ELISA (day 7 for serum IgA, day 14 for intestinal IgA, and day 10 for milk IgA). Furthermore, unlike the ELISA results, intestinal IgG production also seemed to occur at the same time as intestinal IgA production. This discrepancy in the time course of IgA and IgG antibody detection may have been due to differences in the sensitivity of the two methods or in the nature of the target antigens. Indeed, whereas whole *T. gondii* cells were used as antigens for the ELISA, the target antigen in Western blotting was a *T. gondii* SDS extract. However, the Western blots results were similar to those of the ELISA in terms of the kinetic patterns of the IgA, IgG, and IgM responses. The kinetics of the serum IgA antibody response were in agreement with those observed by Le Fichoux et al. (20), who used an immunosorbent agglutination assay to detect mouse serum anti-*T. gondii* IgA antibodies. In contrast, intestinal IgA antibodies were detected earlier than reported by McLeod and Mack (23), who also used an ELISA. Moreover, these authors found that the intestinal IgA antibody response ceased after 7 weeks of infection (23), whereas we were able to detect IgA production until the end of the experiment (17 weeks after infection). One must bear in mind that the antibody response can vary with the experimental infection procedure (e.g., animals, strain virulence, dose, route, and timing of infection). In our case, infection was induced orally with a single dose of 40 strain 76K cysts, a dose which seems to approximate most closely a natural infection, whereas
McLeod and Mack (23) gave three oral doses of strain Me49 cysts at a greater concentration than that used here. The presence of intestinal, milk, and serum IgA antibody responses to T. gondii was also demonstrated by Western blots. Serum IgA recognizing p30 in acute and congenital toxoplasmosis (9) or recognizing three major 25-, 35-, and 50-kDa bands in accidental laboratory toxoplasmosis (27) has been described. We present here the first description of specific toxoplasma antigens recognized by milk and intestinal IgA antibodies. The almost identical migration patterns of major well-known toxoplasma antigens probed with MAbs and the major bands recognized by anti-T. gondii antibodies in the three biological compartments suggest that the identities of the 4.5-, 21-, 24-, 28-, 30-, 38-, 55-, and 60-kDa bands may be the 4.5-kDa antigen, p22, p23, the 28-kDa antigen, p30, p43, and the 55- and 60-kDa antigens, respectively. The antigens p22, p23, p30, and p43 are major surface toxoplasma proteins (3), the 4.5-kDa entity appears to be a polysaccharide antigen (32), the 28-kDa antigen is a dense granule protein (J.-F. Dubremetz, submitted for publication), and the 55- and 60-kDa antigens are rhoptry proteins (30). Indeed, several rhoptry antigens are found in the 55- to 60-kDa range (30, 31; C. Leriche and J.-F. Dubremetz, submitted for publication), but the identity of the bands observed in the present study remains to be defined. Most of the antigens recognized by milk, intestinal, and serum IgA antibodies were also detected by IgG antibodies, in agreement with the previous work of Partanen et al. (27) for serum. IgA antibodies recognized the same major antigens in serum, intestinal secretions, and milk; thus, specific antibody production was not confined to one biological compartment. In addition, the humoral response varied considerably from one mouse to another, since not all mice produced antibodies recognizing the entire antigenic repertoire. The use of syngeneic mice may partly resolve this problem. Milk IgA antibodies mainly reacted with antigens comigrating with p30, p43, and an antigen with an apparent molecular mass of 40 kDa, whereas intestinal IgA antibodies recognized antigens comigrating with p22, p23, the 28-kDa dense granule protein, p30, p43, the 55- and 60-kDa rhoptry proteins, and two antigens with apparent molecular masses of 40 (faint band) and 50 kDa. Early infection was associated with IgA antibodies to p30 (milk, intestinal, and serum samples), p43 (milk and serum samples), and rhoptry antigens (intestinal secretions), in agreement with previous reports showing that p30 and p43 seem to be the first proteins recognized by human sera in acute toxoplasmosis (8) and that the anti-p30 IgA antibodies are useful human serum markers in congenital and acute toxoplasmosis (9).

These initial studies of the humoral immune responses against T. gondii point to a close relationship between the systemic and mucosal immune systems during toxoplasmosis. It will be of great interest to differentiate the two types of IgA antibodies (serum and secretory) in milk and intestinal secretions following T. gondii infection. Moreover, following identification of the toxoplasma antigens recognized by the local immune system, particularly by IgA antibodies, the next step will be the molecular characterization of such
antigens through gene cloning. Characterization of the role of these toxoplasma antigens in local protective immunity is a prerequisite for future oral vaccination studies.

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LITERATURE CITED