INFECTION AND IMMUNITY, Mar. 1989, p. 668–672
0019-9567/89/030668-05$02.00/0

Vol. 57, No. 3

Identification of Stage-Specific Antigens of *Toxoplasma gondii*

LLOYD H. KASPER

Department of Medicine (Neurology) and Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03756

Received 20 September 1988/Accepted 3 December 1988

An immunologic evaluation of the surface antigens of the three major life-cycle stages of *Toxoplasma gondii* was performed. Mouse antisera were raised against these stages, which included the oocyst-sporozoite (feline-excreted stage), bradyzoite (chronic tissue cyst stage), and tachyzoite (invasive stage). The antisera were used in an enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis to demonstrate the presence of stage-specific antigens. These antigens were of various molecular weights and were specific to each stage investigated. Cross-reaction studies showed that the mouse antisera recognized commonly shared antigens to at least two of the three stages. A panel of monoclonal antibodies identified specific immune epitopes unique to each of the stages investigated. These studies further support the hypothesis that stage-specific antigens are present in *T. gondii*.

Although the morphologic characteristics of the life-cycle stages of *Toxoplasma gondii* have been well documented, a detailed immunologic analysis has not been reported (1, 2, 9, 11). We have previously described the identification of stage-specific oocyst-sporozoite antigens of *T. gondii* by monoclonal antibodies (3). These monoclonal antibodies identified at least two unique oocyst-sporozoite antigens that were not expressed on the surface of the tachyzoite. Recognition of stage-specific oocyst-sporozoite antigens also has been demonstrated by using human sera from an epidemiologically well-documented outbreak of oocyst-transmitted infection (6).

In immunofluorescence studies, bradyzoites (cystozoites) have also been found to exhibit stage specificity (8). Similarly, we have shown that some monoclonal antibodies directed at tachyzoite antigens are nonreactive toward bradyzoites (5). Thus, it appears that there are unique antigens that may immunologically characterize the three major morphologic life-cycle stages of toxoplasma. We report here the presence of such stage-specific *T. gondii* antigens.

MATERIALS AND METHODS

Parasites. P-strain parasites were obtained from the Me49 line of *T. gondii* (kindly supplied by M. Lunde, National Institutes of Health) and cloned in our laboratory. This strain produces high numbers of brain tissue cysts in mice and was used for both bradyzoite and tachyzoite preparations. Bradyzoites were produced by infecting mice intraperitoneally with purified P-strain-infected brain cysts and allowing 8 weeks for brain cysts to develop. To purify brain cysts, mice were exsanguinated, and their brains were removed and rinsed in phosphate-buffered saline (PBS). Each brain was homogenized in 2 ml of PBS, and the homogenate was mixed with an equal volume of 30% dextran (M_r, 229,000). This mixture was centrifuged at 1,400 × *g* for 20 min. The pellet was washed once and resuspended in PBS. To prepare bradyzoite lysates, brain cysts were freeze-thawed five times in a dry ice-methanol bath. The suspension was then sonicated eight times (18,000 Hz) at 10-s intervals at 4°C, solubilized by adding Nonidet P-40 to 0.5%, and stored at −20°C.

P-strain tachyzoites were produced by infecting human fibroblast monolayers with P-strain-infected mouse brain tissue that had been homogenized and forcibly passed through a 27-gauge needle. These were maintained as tachyzoites by continuous passage in human fibroblast cells.

Oocysts were obtained in a manner similar to that previously described (9, 10). Briefly, infected mice were sacrificed, and an estimated 1,000 brain cysts were fed to nonimmune kittens. Feces were collected daily and purified by flotation in a sucrose gradient. The oocysts were allowed to sporulate at room temperature for several days in 0.5 N sulfuric acid and stored at 4°C. To prepare oocyst-sporozoite lysates, oocysts were washed several times in Hanks balanced salt solution and freeze-thawed five times in a dry ice-methanol bath. The suspension was then sonicated eight times (18,000 Hz) at 10-s intervals at 4°C, solubilized by adding Nonidet P-40 to 0.5%, and stored at −20°C.

Autoradiogram. Surface antigens of each life-cycle stage were iodinated by the Iodogen method as previously described (14). Bradyzoites were released from purified brain cysts by incubating the cysts with 0.01 M pepsin in 0.01 N HCl for 1 min and syringing the bradyzoites through a 27-gauge needle. Sporozoites were released from oocysts by mechanical fracture. Tachyzoites were released from host cells by forced extrusion through a 27-gauge needle. Iodinated samples were run on a 12.5% polyacrylamide gel, stained, dried, and autoradiogramed for 2 weeks with a Cronex Lightning-Plus intensifying screen.

Preparation of mouse antitoxoplasma antisera. BALB/c mice (Jackson Laboratory) were immunized in three doses with sonicated and freeze-thawed parasites of each life-cycle stage of *T. gondii*. Freund complete adjuvant was used in the first dose and Freund incomplete adjuvant was used for the remaining two doses. Mice were bled 10 days after the final dose. The pooled sera from at least three mice was used as the source of antiserum.

ELISA. Antitoxoplasma antibody was measured by an enzyme-linked immunosorbent assay (ELISA). Purified parasites were placed in microdilution plates, dried overnight, and blocked with 5% fetal bovine serum–PBS. Antisera were incubated for 2 h at 37°C, and the plates were washed in 0.05% Tween 20–PBS. Antibody binding was identified with peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) or IgM (Boehringer Mannheim Biochemicals).

Polyacrylamide gel electrophoresis. Parasite samples were dissolved in sample buffer that contained 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. After being boiled for 3 min, samples were electrophoresed in a 0.1% sodium dode-

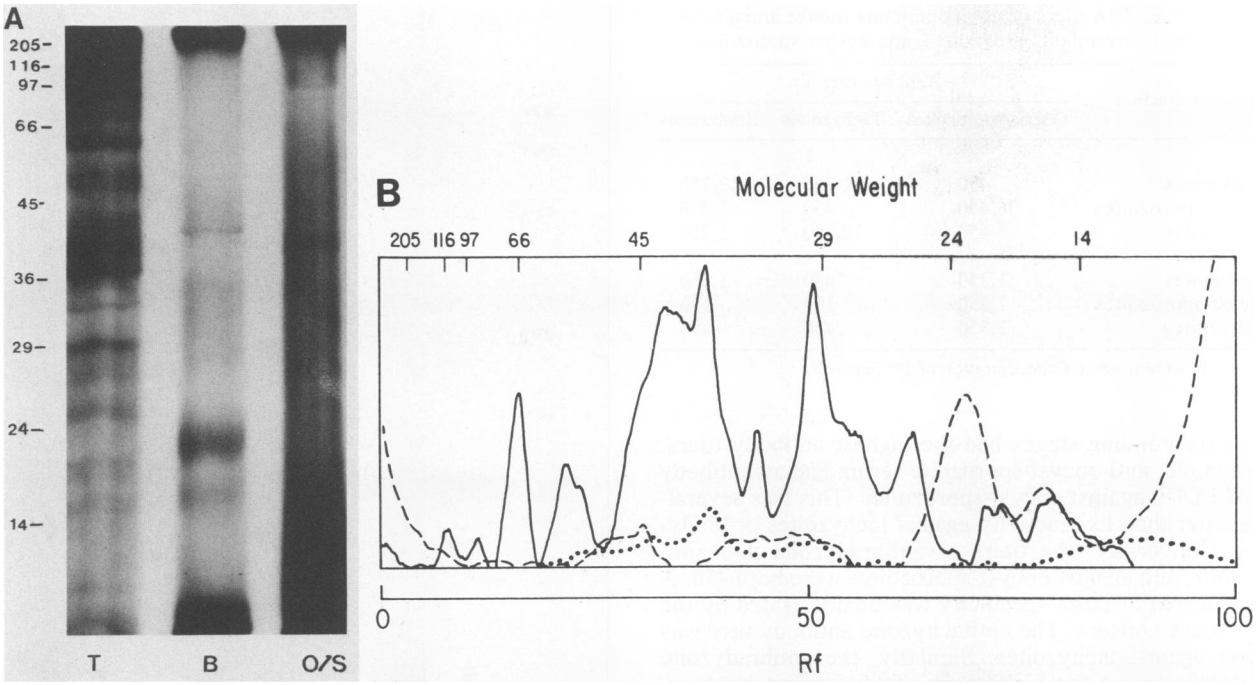

FIG. 1. (A) Autoradiogram of iodinated parasites of the three life-cycle stages of *T. gondii*. Parasites were purified and iodinated by the Iodogen method, run on a 12.5% polyacrylamide gel, and autoradiogramed with a Cronex Lightning-Plus intensifying screen. This autoradiogram represents equal numbers of tachyzoites (T), bradyzoites (B), and oocyst-sporozoites (O/S) run in parallel and autoradiogramed for 2 weeks. Molecular weight standards (10^3) are indicated. (B) Analysis of the lanes in panel A with a videodensitometer (model 620; Bio-Rad Laboratories). ——, Tachyzoites; ------, bradyzoites; ....., oocyst-sporozoites. Base-line values have been subtracted for standardization.

cyl sulfate discontinuous 12.5% polyacrylamide gel. Molecular weight standards included β-galactosidase (116,000), phosphorylase *b* (97,000), bovine albumin (66,000), egg albumen (45,000), carbonic anhydrase (29,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300).

Nitrocellulose blot transfer electrophoresis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gels were applied to a sheet of nitrocellulose paper (0.2-μm pore size) and electrophoresed for 2 h with a Transfor apparatus (13). After electrophoresis, the remaining protein-binding sites on the nitrocellulose paper were blocked with 3% bovine serum albumin in PBS (pH 7.4) overnight at 4°C. Strips were then incubated with appropriate antisera diluted 1:200 in 3% bovine serum albumin–PBS for 2 h at room temperature. The nitrocellulose was washed several times in 0.05% Tween 20–PBS and incubated with peroxidase-labeled goat anti-mouse IgG or IgM (diluted 1:5,000). The strips were washed and developed in a solution of 0.02% 4-chloro-1-naphthol–0.1% hydrogen peroxide in PBS.

Monoclonal antibodies. Production of bradyzoite-specific monoclonal antibodies was accomplished by using our previously reported murine hybridoma method (3, 4). Briefly, mice were immunized over a 3-week period with a total of 1.5×10^7 bradyzoite parasites. Immunization was performed weekly, with injection 1 made in the presence of Freund complete adjuvant and injections 2 and 3 with Freund incomplete adjuvant. Immunized mice were sacrificed 1 week after the final immunization booster, the spleen was removed, and spleen cells were fused with NS-1 myeloma cells in a standard fusion protocol. Culture supernatant from confluent wells was tested for antibradyzoite antibody by ELISA. Positive wells were cloned by limiting dilution, expanded in culture, and used to produce ascites in pristane-pretreated mice.

RESULTS

Radioiodination was performed to determine whether *T. gondii* exhibits stage-specific surface membrane proteins. Both bradyzoites and oocyst-sporozoites were released from their cyst walls and radioiodinated by the Iodogen technique. Tachyzoites were obtained from tissue culture and radiolabeled. Equal numbers of parasites were run in parallel on 12.5% sodium dodecyl sulfate-polyacrylamide gel and autoradiogramed. There were radioiodinatable proteins of different and common molecular weights in each life-cycle stage (Fig. 1A). Both stage-specific and shared iodinated parasite proteins could be seen at different molecular weights. Analysis by videodensitometry showed the relation among these radioiodinated parasite proteins from the three stages (Fig. 1B). Similar results were obtained when equal numbers of radioactive counts for each stage were compared by gel electrophoresis and autoradiography (not shown).

To better determine stage specificity, mouse antitoxoplasma sera were examined by ELISA for the presence of antibodies to bradyzoites, tachyzoites, and oocyst-sporozoites. We have shown previously that tachyzoites and sporozoites bind with equal affinity to a solid substrate (6). To demonstrate that the bradyzoites also adhere, equal numbers (5×10^5) of bradyzoites, tachyzoites, and sporozoites were purified, radioiodinated by the Iodogen method, and deposited in microdilution plates. The plates were blocked in 5% fetal bovine serum, washed, and counted. Similar results were obtained when an equal number of counts (50,000 cpm per well) was used. By this method, it was determined that bradyzoites bind to microdilution plates as well as tachyzoites and sporozoites do.

The results of the ELISA for antibody stage specificity are shown in Table 1. In general, mouse antisera raised against

TABLE 1. ELISA titers of pooled immune mouse antisera to toxoplasma tachyzoites, bradyzoites, and oocyst-sporozoites[a]

Antiserum fraction	Antibody titer for:		
	Oocyst-sporozoites	Tachyzoites	Bradyzoites
IgG			
Bradyzoites	450	1,350	12,150
Oocyst-sporozoites	36,450	450	1,350
Tachyzoites	450	12,150	1,350
IgM			
Bradyzoites	1,350	450	1,350
Oocyst-sporozoites	1,350	150	150
Tachyzoites	1,350	450	450

[a] Parasites were used at a concentration of 10^4 per well.

their corresponding stages had the highest antibody titers. For example, anti-oocyst-sporozoite serum had an antibody titer of 36,450 against oocyst-sporozoites. This was several-fold greater than its reactivity against tachyzoites or bradyzoites. Conversely, the titers of antibradyzoite and anti-tachyzoite sera against oocyst-sporozoites were both 450. A similar degree of stage specificity was demonstrated by the other mouse antisera. The antitachyzoite antibody titer was greatest against tachyzoites. Similarly, the antibradyzoite sera demonstrated the highest specificity against bradyzoites. Both of these sera failed to distinguish between the noncorresponding stages. These differences in titer are probably not due to strain variation. We have previously shown that the ELISA is of insufficient sensitivity to distinguish among strains when strain-specific rabbit antisera are used (14). Results similar to those shown in Table 1 were obtained when the ELISA was run with 0.5 μg of parasite protein per well rather than with equal numbers of parasites.

The IgM fraction of the stage-specific antisera was quantitatively and specifically less than the IgG fraction. There was low-titer cross-reactivity between the three life stages and their noncorresponding antisera (Table 1). The highest specific IgM antibody response was directed against the bradyzoite by its corresponding antiserum.

Further analysis of the stage-specific antigens was done by Western blot (immunoblot). We have demonstrated previously that this technique is of adequate sensitivity to discern strain-specific tachyzoite antigens of *T. gondii* (14). For Western blot analysis, equal numbers of parasites were analyzed by using the stage-specific mouse antitoxoplasma sera. There were antigens unique to each of these *T. gondii* stages (Fig. 2). The arrowheads in Fig. 2 indicate a number of parasite antigens that appear to be unique to that stage or perhaps present in quantitatively greater amounts than in the other stages. In the normal brain tissue control, two low-molecular-weight bands (<14,000) were recognized by the antibradyzoite serum. One band (>97,000) was recognized by the oocyst-sporozoite antiserum in sucrose gradient-purified cat fecal material. There was no recognition of normal human fibroblasts by the antitachyzoite serum. A Western blot of the IgM fractions of the three mouse antisera against their corresponding antigens failed to identify stage-specific antigens that differed from those identified by the IgG fraction (not shown).

A comparative Western blot analysis was performed to determine if there are commonly shared antigens among these three morphologic stages of *T. gondii*. This study was done by evaluating equal numbers of parasites run in parallel and then reacted with the three stage-specific mouse antisera. Several parasite antigens appeared to be commonly

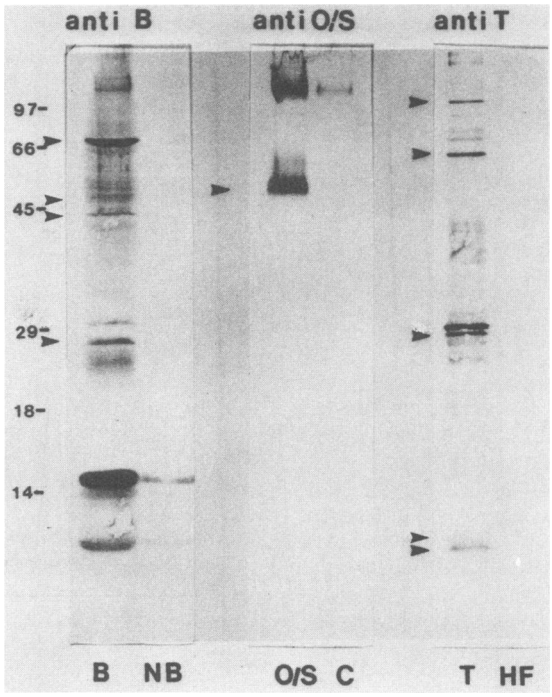

FIG. 2. Comparison of antigens of the three major life-cycle stages of *T. gondii*. Equal numbers (1.8×10^6 parasites per lane) of toxoplasma bradyzoites (B), oocyst-sporozoites (O/S), and tachyzoites (T) were run on 12.5% polyacrylamide gel, transferred to nitrocellulose paper, and reacted with corresponding mouse antisera and assayed for IgG binding. Controls represent equal amounts of protein from normal mouse brain purified by a dextran gradient for bradyzoites (NB), cat fecal material purified by a sucrose gradient (C), and uninfected human fibroblasts (HF). Arrowheads indicate some of the stage-specific antigens. Molecular weight standards (10^3) are indicated.

identified by the bradyzoite and tachyzoite mouse antisera (Fig. 3). Most noteworthy is the cluster of tachyzoite and bradyzoite antigens with M_rs of approximately 24,000 to 32,000, designated the P30 cluster (Fig. 3). There does not appear to be recognition of antigens of this molecular weight in the oocyst-sporozoite preparation. Curiously, the anti-oocyst-sporozoite serum identified one of these antigens in the tachyzoite preparation (as a doublet of approximate M_r 30,000) and in the bradyzoite preparation (as a single band of approximate M_r 24,000) but not in its corresponding oocyst-sporozoite preparation.

The best test for stage specificity is analysis with monoclonal antitoxoplasma antibodies. Monoclonal antibodies have been raised against the tachyzoite and oocyst-sporozoite stages (3, 4). The antitachyzoite monoclonal antibodies used in this experiment were directed against the major iodinatable surface membrane proteins, P30 and P22 (M_rs, 30,000 and 22,000, respectively). Both of these IgG monoclonal antibodies are able to immunoprecipitate their corresponding antigens from radiolabeled, detergent-solubilized tachyzoites. The anti-oocyst-sporozoite IgG monoclonal antibodies have been previously described (3). To identify stage-specific bradyzoite antigens, we developed a panel of murine monoclonal antibodies against the bradyzoite stage. The antibradyzoite monoclonal antibodies are of the IgM isotype and do not immunoprecipitate or identify bradyzoite antigens by Western blot analysis under denaturing conditions. Each of these monoclonal antibodies exhibited a titer

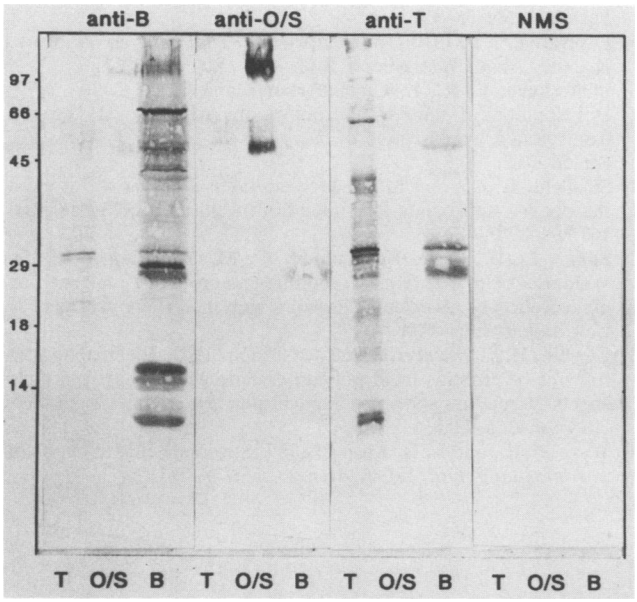

FIG. 3. Western blot analysis of commonly shared and cross-reacting antigens of the three major life-cycle stages of *T. gondii*. Equal numbers (1.8×10^6 parasites per lane) of toxoplasma were run on 12.5% polyacrylamide gel, transferred to nitrocellulose paper, reacted with appropriate mouse antitoxoplasma sera (antibradyzoite [anti-B], anti-oocyst-sporozoite [anti-O/S], and antitachyzoite [anti-T]) or control serum (pooled normal mouse sera [NMS]), and assayed for IgG binding. Molecular weight standards (10^3) are indicated.

of ≥50,000 against its corresponding stage (Table 2). This was a ≥100-fold increase over titers of the noncorresponding stages.

DISCUSSION

Our results show that there are *T. gondii* antigens unique to the oocyst-sporozoite, tachyzoite, and bradyzoite stages of this organism. These antigens can be identified by several immunologic assays that use monoclonal and polyclonal antibodies raised against each of these stages. The identification of these unique antigens confirms that immunologic as well as morphologic stage specificity of *T. gondii* exists.

The most conclusive data in support of stage specificity concern the immunoreactivity demonstrated by monoclonal antibodies raised against each of the three stages. These antibodies were able to distinguish their corresponding stages far better than they could distinguish noncorresponding stages. For example, monoclonal antibodies raised against two major tachyzoite antigens (P30 and P22) were 100-fold more reactive with the tachyzoite stage than with the oocyst-sporozoite and bradyzoite stages. Similarly, high-titer (≥50,000) anti-oocyst-sporozoite monoclonal antibodies reacted poorly with bradyzoites and tachyzoites. The development of an antibradyzoite monoclonal antibody library supports previous suggestions that antigenic specificity of this stage exists (8, 9). Because current epidemiologic evidence suggests that acute toxoplasmosis in patients with acquired immune deficiency syndrome may represent recrudescence of previous infection (7), the identification, characterization, and purification of bradyzoite antigens may in the future be of clinical importance in the serodiagnosis of toxoplasmosis.

TABLE 2. ELISA titers of stage-specific monoclonal antibodies reactive with the three major life-cycle stages of *T. gondii*[a]

Antibody	Antibody titer for:		
	Oocyst-sporozoites	Tachyzoites	Bradyzoites
Antibradyzoite			
4B5	500	500	≥50,000
3E9	50	50	≥50,000
Antisporozoite			
2E4	≥50,000	50	500
5A4	≥50,000	Neg[b]	Neg
Antitachyzoite			
7B8	50	≥50,000	500
7G1	Neg	5,000	50

[a] Parasites were used at a concentration of 10^4 per well.
[b] Neg, Negative.

Further support for stage specificity of *T. gondii* antigens was shown by ELISA and Western blot analysis. Despite cross-reactivity among the various stages, the ELISA was sufficiently sensitive to distinguish stage-specific antigens. In general, the IgM titer was lower and less specific than the IgG titer. The Western blot also distinguished stage-specific antigens. Several of these antigens were not easily detected by radioiodination, suggesting that they were not labeled by the Iodogen technique (thus being tyrosine deficient) or, more likely, that some of the immunoreactive epitopes are not proteins.

The identification of antigens shared among the three stages of *T. gondii* is also important. Those antigens which are recognized by their cross-reactivity with noncorresponding antisera may be of either membranous or cytoplasmic origin. They may reflect a common biochemical thread that runs through the rather complicated two-host life cycle of this parasite. It is possible that some of the higher-molecular-weight antigens are precursors of lower-molecular-weight parasite membrane proteins.

Of particular interest is the P30 cluster of immunoreactive proteins between M_rs 24,000 and 34,000. P30 is a major tachyzoite membrane protein and is a component of the vesicular network found within the parasitophorous vacuole (12, 14). It is highly immunoreactive in Western blots with the wild-type P or C strain but not with the RH strain. Preliminary data from Western blot analysis by our laboratory suggest that the immunoreactive molecules of P30 may be glycosylated (unpublished data). Our results show that the antisera raised against the bradyzoite and tachyzoite stages of *T. gondii* contain antibodies that react with proteins in the P30 cluster. Further analysis of the bradyzoite and tachyzoite proteins within the P30 cluster is under way; an understanding of these proteins may be important in improving both serodiagnosis of and immunization against toxoplasmosis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-19613 from the National Institutes of Health and by the March of Dimes Birth Defects Foundation.

I thank Patricia Ware for her technical assistance.

LITERATURE CITED

1. **Frenkel, J. K., J. P. Dubey, and N. L. Miller.** 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. Science **167**:893–896.
2. **Hutchinson, W. M., J. F. Duncachie, J. C. Siim, and K. Work.**

1970. Coccidian-like nature of *Toxoplasma gondii*. Br. Med. J. **1**:142–147.
3. **Kasper, L. H., M. S. Bradley, and E. R. Pfefferkorn.** 1984. Identification of stage specific sporozoite antigens of *Toxoplasma gondii* by monoclonal antibodies. J. Immunol. **132**: 443–449.3.
4. **Kasper, L. H., J. H. Crabb, and E. R. Pfefferkorn.** 1982. Isolation and characterization of a monoclonal antibody resistant mutant of *Toxoplasma gondii*. J. Immunol. **129**:1694–1699.
5. **Kasper, L. H., K. M. Currie, and M. S. Bradley.** 1985. An unexpected response to vaccination with a purified major membrane tachyzoite antigen (P30) of *T. gondii*. J. Immunol. **134**: 3426–3432.
6. **Kasper, L. H., and P. L. Ware.** 1985. Recognition and characterization of stage-specific oocyst/sporozoite antigens of *Toxoplasma gondii* by human antisera. J. Clin. Invest. **75**:1570–1577.
7. **Luft, B. J., and J. S. Remington.** 1988. Toxoplasmic encephalitis. J. Infect. Dis. **157**:1–6.
8. **Lunde, M. N., and L. Jacobs.** 1983. Antigenic differences between endozoites and cystozoites of *Toxoplasma gondii*. J. Parasitol. **69**:806–811.
9. **Overdulve, J. P.** 1970. The identity of *Toxoplasma gondii*. Proc. K. Ned. Akad. Wet. Ser. C Biol. Med. Sci. **73**:129–135.
10. **Pfefferkorn, E. R., L. C. Pfefferkorn, and E. D. Colby.** 1977. Development of gametes and oocysts in cats fed cysts derived from cloned trophozoites of *Toxoplasma gondii*. J. Parasitol. **63**:158–165.
11. **Sheffield, H. G., and M. L. Melton.** 1970. *Toxoplasma gondii*: the oocyst, sporozoite and infection of cultured cells. Science **167**:892–896.
12. **Sibley, L. D., J. D. Krahenbuhl, G. M. W. Adams, and E. Weidner.** 1986. *Toxoplasma* modifies macrophage phagosomes by secretion of a vesicular network rich in surface proteins. J. Cell Biol. **103**:867–874.
13. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4356.
14. **Ware, P. L., and L. H. Kasper.** 1987. Strain-specific antigens of *Toxoplasma gondii*. Infect. Immun. **55**:778–783.