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Comparative Study of Tissue Culture and Mouse Inoculation Methods for Demonstration of *Toxoplasma gondii*

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Two methods for the isolation of *Toxoplasma gondii* were analyzed and compared. Bradyzoites or tachyzoites of three strains of *T. gondii* were injected into mice and introduced in parallel onto MRC5 fibroblasts cultured on cover slips. In the cultures, the parasites were more readily identified by an indirect immunofluorescence assay than by examination of unstained or Giemsa-stained cultures. With the RH strain, the tachyzoites replicated actively, and large foci of parasites were observed in 24 h. The bradyzoites or tachyzoites of the other strains could also be cultivated, but grew rather slowly; 2 days after inoculation, early stages of multiplication could be observed: from day +4, *Toxoplasma* clusters or foci were easily identified at a ×100 magnification. The course of infection in mice was greatly dependent on the virulence of the strain and on the parasitic stage inoculated. In the chronically infected mice, evidence of *Toxoplasma* infection was only detected 45 days after inoculation through the demonstration of cysts in the brain or the presence of specific antibodies in the serum. The mean ratio of infected mice and positive cultures was compared in relation to the inoculum size. The tissue culture method was found to be at least as sensitive as mouse inoculation. Since *Toxoplasma* organisms may be isolated within a few days in tissue culture, it is proposed that this method should be used when early isolation of the parasite is crucial for the diagnosis of toxoplasmosis.

Toxoplasma gondii is usually isolated from susceptible laboratory animals. Mice are most commonly used, since they can be readily infected by intraperitoneal injections of trophozoites or bradyzoites. Depending on the virulence of the strain, mice develop either an acute infection with parasite-rich ascites or a chronic infection characterized by the presence of cysts in the brain.

Inoculation of blood, body fluids, or tissue extracts into mice may be used for diagnosis (1, 12), but the result is often delayed because the encysted parasites may not be identified before 30 days after inoculation.

Several reports have demonstrated that tissue culture methods could be applied to the rapid isolation of *Toxoplasma* organisms from blood (7, 17) or infected tissues (2, 5), and could serve for diagnosis when serological tests are inconclusive. However, the sensitivity of these methods has not been evaluated. This was the main objective of this study, in which three strains of *T. gondii* were cultivated on human fibroblasts (MRC5 strain) and inoculated into mice.

MATERIALS AND METHODS

Mice. Adult male and female Swiss albino mice were used. All were negative for anti-*Toxoplasma* antibodies in the direct agglutination test (Bio-Mérieux, France).

Parasites. Three strains of parasites were studied. The RH virulent strain was maintained in mice by syringe passages of peritoneal fluid from infected mice at 3-day intervals. The two other strains were of human origin; one (C strain) was obtained from a congenitally infected placenta (5), and the other (H strain) was isolated from the blood of a bone marrow transplant patient with disseminated toxoplasmosis. These strains were of low virulence in mice and could be maintained through passage at 6-month intervals by intraperitoneal inoculation of brain cysts. To obtain tachyzoites, cysts from chronically infected mice were inoculated intraperitoneally into mice treated with cortisone acetate (0.2 mg/day subcutaneously from the day of inoculation); peritoneal exudate containing tachyzoites was harvested 5 to 7 days later.

For the collection of tachyzoites, the peritoneal cavity of infected mice was washed with 5 ml of sterile phosphate buffer solution (PBS; 0.01 M, pH 7.2). Contaminating cells were eliminated by gel filtration through Trisacryl GFO5 (IBF-France); 10 ml of gel was packed into a 20-ml syringe barrel and washed with 40 ml of PBS. An equal volume of peritoneal wash collected from infected mice was allowed to pass through the column; the filtrate contained tachyzoites with less than 0.01% contaminating peritoneal cells.

Bradyzoites (C and H strains) were obtained from chronically infected mice. Cysts were isolated from brain tissues on a Percoll gradient (Pharmacia, Sweden) (3) and then disrupted by trypsin digestion (trypsin 1/250 [Difco Laboratories]; 0.5% in PBS, 5 min at 37°C). Bradyzoites were centrifuged and washed with PBS.

Viable tachyzoites and bradyzoites (brightly refringent by phase-contrast microscopy) were suspended in minimum essential medium (MEM) (Flobio, France) supplemented with 10% fetal calf serum (Flow Laboratories, France) and enumerated in a hemacytometer. They were inoculated into mice or cultures within 2 h after collection.

Toxoplasma antigen. Toxoplasma antigen was prepared from *T. gondii* tachyzoites of the RH strain. Purified parasites were lysed with distilled water and then disrupted by six successive cycles of freezing and thawing. After centrifugation at 10,000 × *g* for 1 h, the supernatant fluid was collected and used as the antigen for immunization of rabbits. The protein content was 0.5 mg/ml (13).

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***Toxoplasma* antibodies.** Antibodies to *T. gondii* were obtained by immunization of New Zealand White rabbits. Initial immunization was made with 500 μg of antigen mixed with 0.5 mg of muramyl dipeptide (Choay Chimie, France) and 1 ml of incomplete Freund adjuvant (Difco); 200 μl was injected intradermally at five sites. An identical weekly booster was administered for 2 months until a serum agglutination titer of 1:4,096 was obtained. The rabbits were exsanguinated, and immunoglobulin G (IgG) was purified by ion-exchange chromatography (DEAE Trisacryl; IBF, France) (16).

Cell cultures. Human embryonic fibroblast cell line MRC5 (Bio-Mérieux, France) was maintained in MEM containing 10% decomplemented fetal calf serum, kanamycin (200 μg/ml), and ampicillin (100 μg/ml). One milliliter of a suspension containing 10^5 fibroblasts was seeded onto 12-mm-diameter round cover slips (GIBCO Laboratories) placed into each well of 24-well plates (Nunc, Sweden). Cultures were incubated at 37°C in a moist 5% CO_2–95% air atmosphere and used within 2 weeks after preparation.

Identification of *T. gondii* in cell cultures. Three methods were used to identify *T. gondii*: (i) direct examination with an inverted microscope (×250); (ii) examination of Giemsa-stained cultures (×400); and (iii) indirect immunofluorescence (IF) (15). Cover slip cultures were rinsed with PBS, fixed with cold acetone (15 min at −20°C), and then incubated in the wells for 30 min at 37°C with antitoxoplasma antibodies diluted at 1/200 in PBS. After two washes with PBS, a fluorescent anti-rabbit IgG (Institut Pasteur, France) diluted at 1/100 in PBS containing 1/25,000 Evans blue was added. After 30 min at 37°C, the cover slips were washed and mounted onto slides for examination with an Olympus BH2 microscope with a reflected-light fluorescent illuminator (light source, HBO 100 W; excitation filter, 490 nm; barrier filter, 515 nm).

A comparative evaluation of the three methods was made with cultures inoculated with 10^3 tachyzoites or bradyzoites from the different strains. In the cultures inoculated with the virulent RH strain, the medium was changed after an initial contact of 3 h with the parasites, and then two cover slips were examined; other cultures were maintained at 37°C and examined at 6, 9, and 12 h and 1, 2, 4, and 8 days after inoculation (two cover slips). In the cultures inoculated with the low-virulence C and H strains, the medium was changed at day +1 in all the wells, and two cover slips were examined. Other examinations were done after 2, 4, 8, and 10 days.

Comparison of culture and mouse inoculation. Suspensions of tachyzoites of the RH, C, and H strains and bradyzoites of the C and H strains containing 1, 3, 10, 30, and 100 parasites were prepared. Each suspension was inoculated simultaneously into four cell culture wells (1 ml per well) and into each of two mice (1 ml, intraperitoneally). Four control mice were injected with MEM alone.

All assays were repeated in a separate experiment (in one of the two assays with RH tachyzoites, only one mouse was used for each inoculum dose). For each suspension, one of the four cover slip cultures was examined by IF as already described on days 2, 4, 8, and 10.

Inoculated mice were followed up for 45 days. Any animal dying during this period was examined for the presence of toxoplasmas in peritoneal exudate or in the brain; serological tests were not performed. Surviving mice were sacrificed at day 45 and tested serologically by the agglutination test (Bio-Mérieux) and the IF antibody test with fluorescent anti-mouse immunoglobulin (Institut Pasteur, France). The presence of brain cysts was determined by examination of six brain fragments (one frontal and two parietal fragments of each hemisphere).

Statistical analysis. The effects of method (mouse inoculation versus culture), strain, and inoculum dose were evaluated in a maximum likelihood logistic regression analysis of the number of positive mice per the total number of inoculated mice or cultures (4).

Correlations between the number of positive brain smears or cultures and the number of parasites inoculated were studied by the nonparametric Spearman test (R) (8).

RESULTS

Development of toxoplasmas in culture. Tachyzoites and bradyzoites from all strains could be grown in the fibroblast cultures. Tachyzoites of the RH strain replicated in the cells within 6 to 9 h. By 12 h, parasites often formed rosettes or dispersed into the cytoplasm of the host cells. No limiting membrane was observed by IF or Giemsa staining. At 48 h, most of the host cells were infected and some of them detached from the cover slips; at 96 h, monolayers were destroyed and large numbers of tachyzoites were found in the supernate.

Tachyzoites and bradyzoites of the H and C strains grew rather slowly; no marked difference was noticed in development in culture between the two strains. The early stages of multiplication were observed during days 1 and 2. IF staining caused the entire cytoplasm of some infected cells to fluoresce. At days 4 and 8, the parasites formed compact clusters limited by a thin fluorescent membrane. On day 10, some pseudocysts (clusters) had burst, and large foci of newly infected cells were seen.

Comparison of methods for identification of toxoplasmas. By direct examination (×250), a cytopathic effect was detected after 24 to 48 h in culture with the RH strain and on day 4 with the other strains. It presented as foci of fibroblasts with a granular appearance, but *Toxoplasma* organisms could not be identified. Thereafter, pseudocysts were observed as enlarged fibroblasts containing refringent inclusions. Large typical cysts were rare. Usually, cytopathic effects could not be differentiated from nonspecific degenerative changes of the monolayers.

Giemsa staining of the cultures allowed a better analysis of the structure of both parasites and hosts cells; however, scarce parasitized cells were difficult to detect in the stained monolayers.

By IF, parasites could be identified at the early stages of multiplication. At a magnification of ×100, the infected cells could be detected, and *Toxoplasma* organisms were unequivocally identified when examined at ×400. Since this method was found to be more sensitive for identification of *Toxoplasma* organisms in cultures, it was used for the comparative study of the sensitivity of tissue culture versus mouse inoculation.

***Toxoplasma* infection in mice.** Mice injected with tachyzoites of the RH strain died of acute infection within 9 days and presented parasite-rich ascites. With the low-virulence H strain, the course of infection was dependent on the parasite stage inoculated: mice infected with tachyzoites died of acute infection, but very few parasites were found in the peritoneal exudate at the time of death; mice infected with bradyzoites developed chronic infection. With the avirulent C strain, mice developed chronic infection whether infected with tachyzoites or bradyzoites. A total of 29 mice were chronically infected (18 with the C strain and 11 with

the H strain). In 28 of 29 mice, cysts were found in at least one brain smear; for one mouse, six brain smears were negative but blind passage was positive. Brain cysts were found either grouped or dispersed and ranged in size from 15 to 100 μm in diameter. The number of positive smears was found to correlate poorly with inoculum size ($R = 0.38$, $P < 0.05$) (Fig. 1).

The immunofluorescent antibody test was strongly positive (titers of >1:500) for the 29 chronically infected mice; the agglutination test was positive for 28 of 29. For control mice, brain smears and serological tests were negative.

Comparison of sensitivity of the methods. Cultures were examined after 2, 4, 8, and 10 days; for each inoculum size, results included examination of six cover slip cultures inoculated with tachyzoites (three strains in duplicate wells) and four cultures inoculated with bradyzoites (two strains in duplicate wells). The ratio of positive to inoculated cultures was calculated for each inoculum. No significant difference was found when the cultures were examined at each 2-day interval ($P = 0.27$); therefore, mean positive values were calculated from the four determinations (24 cultures with bradyzoites and 16 cultures with tachyzoites for each inoculum size) (Table 1).

One culture of the 24 inoculated with one tachyzoite was positive; however, a significant number of positive cultures were only observed for an inoculum containing more than three bradyzoites or tachyzoites ($P < 0.01$). We noticed that with the low-virulence strains, the host cells examined on day 2 contained only a few parasites; examination of the cultures from day 4 on was easier because parasites formed clusters which could be identified at low magnification (×100).

Mice developed either acute or chronic infections; thus, *Toxoplasma* infection was assessed by a positive serological test or by the presence of parasites in brain biopsies or peritoneal exudates. Following these criteria, positive ratios (i.e., number of mice infected/number inoculated) were calculated for mice inoculated with tachyzoites or bradyzoites (Table 1).

The minimum infecting dose was three tachyzoites or bradyzoites; inoculation of 10, 30, and 100 parasites resulted in an increase in the infection rate (Fig. 2).

Ratios of positive mice and cultures were both significantly related to the inoculum dose ($P < 0.03$) (Fig. 2). No significant difference between the two methods was found ($P = 0.96$), and no difference was observed in the sensitivity of the two methods according to strain or parasite stage inoculated ($P = 0.11$).

TABLE 1. Sensitivity of mouse inoculation and tissue culture to inoculation with tachyzoites and bradyzoites

Parasite stage	No. inoculated	No. of positive cultures/no. inoculated (mean positive ratio)[a]	No. of positive[b] mice/no. inoculated (mean positive ratio)	Organisms found (no. of mice) in: Ascites	Brain cysts
Tachyzoites	1	1/24 (0.04)	0/10 (0)	0	0
	3	4/24 (0.17)	2/11 (0.18)	1	1
	10	11/24 (0.46)	4/11 (0.36)	3	1
	30	18/24 (0.75)	10/11 (0.91)	7	3[c]
	100	22/24 (0.92)	8/11 (0.73)	7	1
Bradyzoites	1	0/16 (0)	0/8 (0)		
	3	5/16 (0.31)	3/8 (0.37)		
	10	12/16 (0.75)	6/8 (0.75)		
	30	14/16 (0.87)	6/8 (0.75)		
	100	16/16 (1)	8/8 (1)		

[a] Mean positive ratio calculated from eight determinations (on days 2, 4, 8, and 10, duplicate wells) for each inoculum level of three strains (tachyzoites) or two strains (bradyzoites).

[b] Mice were considered positive on the basis of a positive serological test or by the presence of parasites in the brain or ascites.

[c] One found positive after blind passage.

DISCUSSION

Animal inoculation is usually considered the most sensitive method for the isolation of *T. gondii* from tissues or body fluids (1, 11). However, several studies have demonstrated that in mice, susceptibility to *Toxoplasma* and course of infection may be affected by several factors, such as the route of infection and the infecting dose (6, 11, 12). In addition, the mouse virulence of the parasite may influence the ability to isolate *Toxoplasma*, since some strains infect mice poorly (10, 14).

The reliability of the mouse model requires regular follow-up studies for the presence of *Toxoplasma* and serological tests. In acutely infected mice, tachyzoites can be found in the peritoneal exudate, but may be rare even at the time of death in mice infected with the H strain. In most instances, mice develop a chronic infection that requires 30 to 45 days. The detection of anti-*Toxoplasma* antibodies by IF is a good indicator of infection because all the chronically infected mice became seropositive while the controls re-

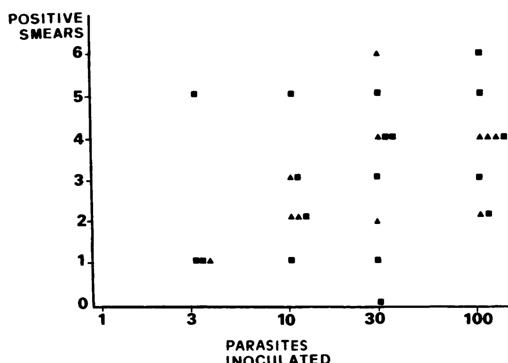

FIG. 1. Relationship between the number of positive brain smears (six examined) and the inoculum size (tachyzoites or bradyzoites) in 29 chronically infected mice. Symbols: ■, C strain (18 mice); ▲, H strain (11 mice).

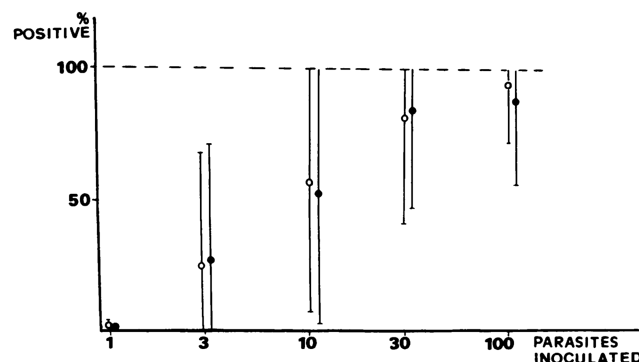

FIG. 2. Relationship between the mean ratio (±1 standard error) of positive culture (○) and mice (●) and the number of bradyzoites or tachyzoites inoculated.

mained negative. However, the demonstration of cysts in the brain provides the best evidence of infection; at least three brain smears should be examined since the cysts may be rare and their number poorly correlated to the inoculum size.

Finally, the methods used for the demonstration of *Toxoplasma* infection in mice can only be applied for routine diagnosis in specialized laboratories; the results are often delayed since mice usually develop a chronic rather than an acute infection. In contrast, tissue culture methods which are commonly used for isolation of viral pathogens are more readily available for the cultivation of *T. gondii* (9).

Our results show that tachyzoites or bradyzoites from different strains of *T. gondii* can be grown in MRC5 fibroblasts cultures. With an IF technique, *Toxoplasma* organisms were easily identified in fibroblasts within 2 days after inoculation; however, later examination (after 4 days) is preferable for easy identification of the parasites in the cultures since large foci or clusters of toxoplasmas can be observed.

The sensitivity of tissue culture and mouse inoculation methods for the demonstration of *Toxoplasma* organisms was compared by using several inocula of bradyzoites and tachyzoites. The two methods were found to be equally sensitive, since the mean positive ratio of infected mice versus that of cultures was not significantly different whether the inoculum was 1 or 100 parasites.

We propose the use of the tissue culture technique for the diagnosis of active toxoplasmosis when serological tests are inconclusive. In congenitally infected children, as well as in immunocompromised patients, rapid demonstration of the presence of *Toxoplasma* organisms in blood or tissue extracts may be crucial for therapy.

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