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Immunohistological Study of the Anatomic Relationship of Toxoplasma Antigens to the Inflammatory Response in the Brains of Mice Chronically Infected with *Toxoplasma gondii*

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The relationship of toxoplasma antigen(s) to the origin and long-term persistence of the mononuclear cell inflammatory infiltrate that is present in the brains of mice chronically infected with *Toxoplasma gondii* was studied by using the peroxidase-antiperoxidase immunohistochemical staining technique. C3H/Km mice were infected with the avirulent C37 strain of *T. gondii* and sequentially sacrificed over the ensuing 107 days. Comparable sections of each brain were prepared for routine light microscopy. Antisera to toxoplasma made in rabbits were used for immunohistological staining, and adjacent slides were also stained with conventional histological stains. The peroxidase-antiperoxidase stain demonstrated toxoplasma tissue cysts, tachyzoites, and intra- and extracellular antigen-antibody reaction products. Early infection was characterized by small tight clusters of free tachyzoites gaining access to brain substance in the absence of an inflammatory response. Once there was disruption of neural parenchyma, a mononuclear cellular infiltrate rapidly ensued. After the first days of infection, mononuclear cells were always present in all infected brains and were anatomically associated with some component of toxoplasma antigen(s). The histological picture of late infection suggested that recurrent episodes of hematogenous dissemination of tachyzoites occurred in infected mice and that such episodes were at least partially responsible for persistence of an antigenic stimulus.

When encephalitis caused by congenital or acquired infection with *Toxoplasma gondii* is clinically apparent, it is usually a devastating disease which leads to death or permanent neurological deficit. However, on the basis of serological testing, it is estimated that in the United States approximately 35% of an asymptomatic population has been infected with toxoplasma (20), and incidental toxoplasma infection of the brain has been discovered at autopsy (19, 28). Chronic infection of the central nervous system (CNS) with this parasite must occur in humans, but little is known about its prevalence, pathogenesis, or long-term effects on brain function. Material is not readily available from human sources to study the course of chronic toxoplasma encephalitis.

By using an avirulent strain of toxoplasma, it is possible to produce a chronic acquired infection in mice which causes no obvious neurological deficit, although a recent study demonstrated that chronically infected mice and rats had learning and memory deficiency when compared with uninfected control animals (30). A mononuclear cell inflammatory reaction which persists for the life of the animal (F. K. Conley, unpublished data) is seen in the brains of mice that are chronically infected with toxoplasma. Previous work from our laboratory has demonstrated that chronically infected mice are able to resist the growth of both intracerebrally implanted tumor (3) and tumor metastatic to the CNS (2). Since mononuclear inflammatory cells are apparently instrumental in effecting this antineoplastic action, it is important to learn more about the causal relationship between chronic toxoplasma infection and the origin and persistence of this cellular infiltrate.

The histological study of chronic toxoplasma encephalitis is made complex by the fact that whereas conventional staining of Formalin-fixed, paraffin-embedded tissue sections readily demonstrates toxoplasma tissue cysts, the proliferative (tachyzoite) form of toxoplasma infection is much more difficult to visualize (6, 11). Of interest is the fact that toxoplasma tissue cysts in infected mouse and human brain are usually situated well outside the areas of inflammatory response and histologically do not appear anatomically related to the population of mononuclear cells in the brain.

We have recently applied the peroxidase-antiperoxidase (PAP) immunohistochemical technique developed by Sternberger (26) to a study

of autopsied human cases of necrotizing toxoplasma encephalitis (F. K. Conley, K. A. Jenkins, and J. S. Remington, Human Pathol., in press) and found that the immunohistochemical method applied to Formalin-fixed, paraffin-embedded tissue sections did not compromise cellular detail, allowed excellent visualization of the tachyzoites as well as of the cysts, and also stained both intra- and extracellular antigenic components derived from the parasite. The present sequential study of chronic infection in mice with *T. gondii* was performed to determine whether some form of toxoplasma antigen source other than from the tissue cyst was anatomically related to the long-term mononuclear cell inflammatory reaction seen in the brain.

MATERIALS AND METHODS

Mice. C3H/Km female mice were obtained from the breeding colony of the Radiobiology Department, Stanford University School of Medicine, and were 8 weeks old at the time of toxoplasma infection.

Infection with *I. gondii*. On day 0, 75 mice received an intravenous injection of 10^5 tachyzoites of the avirulent C37 strain of *T. gondii*. The mice received no antibiotics and were maintained under standard conditions of animal care.

Histological preparations. On days 4 through 11, one or two mice were sacrificed each day, and thereafter two mice were routinely sacrificed every 3 or 4 days by CO_2 inhalation. The last mice were sacrificed on day 107. During the course of the study a normal, uninfected mouse was also sacrificed every 2 weeks to serve as a histology control. Brains from all mice were removed from the cranium and placed in 10% buffered Formalin. After fixation, two comparable coronal sections were cut from the cerebral hemispheres of each brain and processed for routine light microscopy. Sections were cut at 5 μm, and adjacent sections were stained with hematoxylin and eosin (H&E), methyl green pyronin (MGP), and PAP immunohistochemical staining, using a light MGP counterstain (PAP-MGP). This counterstain allowed visualization of cellular detail without obscuring the brown reaction product of the immunological staining and did not by itself stain the tachyzoite form of *T. gondii*. Selected brain sections were also stained with periodic acid-Schiff (PAS) and Giemsa stains.

Histology slides were prepared from a human case of multiple sclerosis and a case of herpes encephalitis. These, along with tissue sections of murine cardiac trypanosomiasis and methanol-fixed smears of *T. cruzi* obtained from the peritoneal cavity of infected mice, were stained by the PAP method, using antitoxoplasma antiserum.

Antisera. Antisera to *T. gondii* were obtained from rabbits which had been infected with *T. gondii* and were kindly provided by Jack S. Remington, Palo Alto Medical Research Foundation. The serological characteristics of these antisera and experiments defining their sensitivity and specificity for staining of *T. gondii* by the PAP method are detailed elsewhere (Conley et al., in press). Normal swine serum, and PAP antiserum were obtained from DAKO-Immunoglobulins, Copenhagen (Accurate Chemical and Scientific Corp., Hicksville, N.Y.), and all lots used were negative for toxoplasma antibody (by the Sabin-Feldman dye test [25]).

Immunohistochemical staining. A modification of the PAP technique developed by Sternberger (26) was used and is fully described elsewhere (Conley et al., in press). The dilutions of antitoxoplasma antisera which were used in this study and which produced positive immunohistochemical staining ranged from 1:100 to 1:10^6.

RESULTS

Infected mice. Mice tolerated infection with the C37 strain of toxoplasma without difficulty and during the course of the study appeared indistinguishable from uninfected control animals. Neurological symptoms such as seizures, lethargy, circling, or focal paralysis were not noted.

General staining characteristics. In the tissue sections from toxoplasma infected mice, toxoplasma tissue cysts could be identified by all the stains used. By H&E, Giemsa, and PAS stains, the cysts had a characteristic speckled appearance. By PAP staining, the cysts stained brown with an irregular pattern of more darkly staining brown on their surfaces; sometimes, if a cyst was cut in true cross section, tightly packed round circles, presumably representing individual organisms, were identified (Fig. 1). Although the proliferative form of toxoplasma, the tachyzoite, was occasionally visualized by H&E, PAS, and Giemsa stains when they occurred in clusters, they could be identified only with certainty by the PAP stain. Evidence of toxoplasma antigen in the cytoplasm of infected cells or residual particulate debris from destroyed toxoplasma organisms also could be vis-

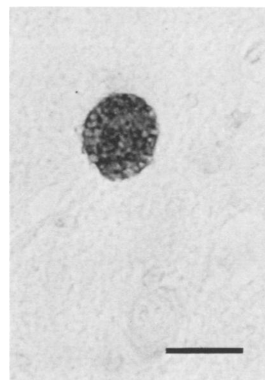

FIG. 1. *Toxoplasma cyst in brain parenchyma 79 days after infection. PAP-MGP, 1:100 dilution of rabbit antitoxoplasma antiserum. Bar equals 17 μm.*

ualized only by the PAP stain. Although inflammatory changes could be appreciated by the MGP counterstain used in conjunction with the PAP stain, they were much more vividly demonstrated with the H&E, PAS, or Giemsa stain.

Immunological staining was absent in the human brain material. There was also no staining of organisms in either tissue sections or smears from mice infected with *T. cruzi* when antitoxoplasma antiserum was used as the primary antibody in the PAP method.

Histology studies. (i) Early infection (0 to 2 weeks). There were no histological changes in the brains 4 days after infection. By day 5, the first tiny aggregate collections of tachyzoites could be seen, often in proximity to a small blood vessel but in the absence of an inflammatory response (Fig. 2). One or two tissue cysts, identified by the H&E stain and no larger than the surrounding glial cells, were seen in association with these tightly packed clumps of tachyzoites (Fig. 3). Over the next 2 days, definite histological abnormalities were present in all brain sections examined. H&E staining revealed tiny focal collections of mononuclear inflammatory cells around small blood vessels; there was no leptomeningeal inflammatory reaction. After day 5, toxoplasma tachyzoites were much more readily identified in tissue sections by the PAP-MGP stain than by any of the other stains. The clumps of tachyzoites were less tightly aggregated, spreading out in a circumferential manner and creating multiple areas of micronecrosis and lacy vacuolation of the neural parenchyma (Fig. 4 and 5). A few mononuclear cells were associated with this process. Infected cells which maintained their distinct cellular morphology and which contained a discrete parasitic vacuole were not seen, but groups of tiny tissue cysts and very occasional unidentifiable cells with amorphous brain-staining antigen-antibody reaction products in their cytoplasm were observed (Fig. 6). By day 11 after infection, histological abnormalities were much more pronounced. Mononuclear inflammatory cells were gathered into microglial nodules in the neural parenchyma and also formed asymmetric collections in the leptomeninges. The PAP stain demonstrated that focal collections of free tachy-

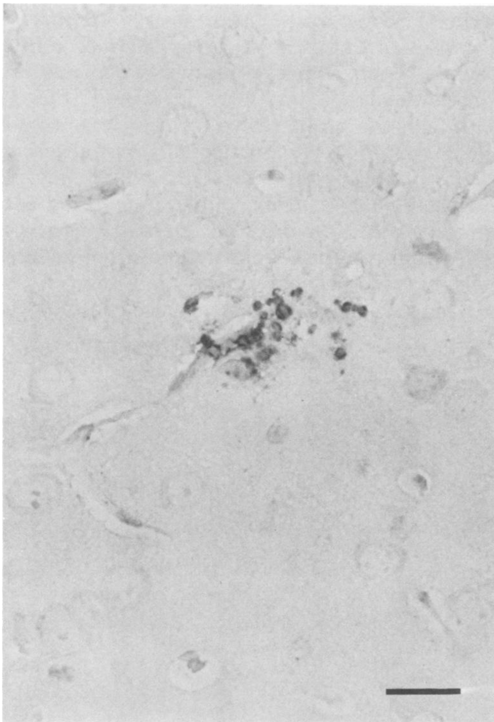

FIG. 2. *Small focus of toxoplasma tachyzoites surrounding a blood vessel 5 days after infection. PAP-MGP, 1:1,000 dilution of rabbit antitoxoplasma antiserum. Bar equals 20 μm.*

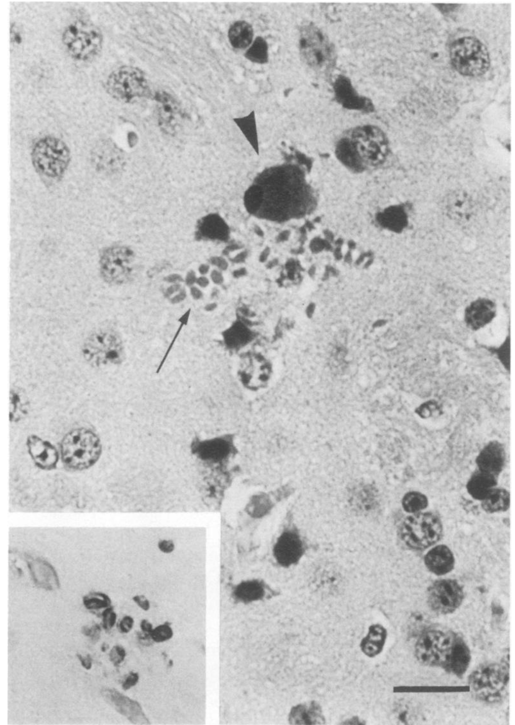

FIG. 3. *Small focus of toxoplasma tachyzoites (small arrow) and early tissue cyst (arrowhead) 5 days after infection. H&E stain. (Inset) Small focus of tachyzoites 5 days after infection stained by PAP-MGP, using a 1:1,000 dilution of rabbit antitoxoplasma antiserum. Bar equals 20 μm.*

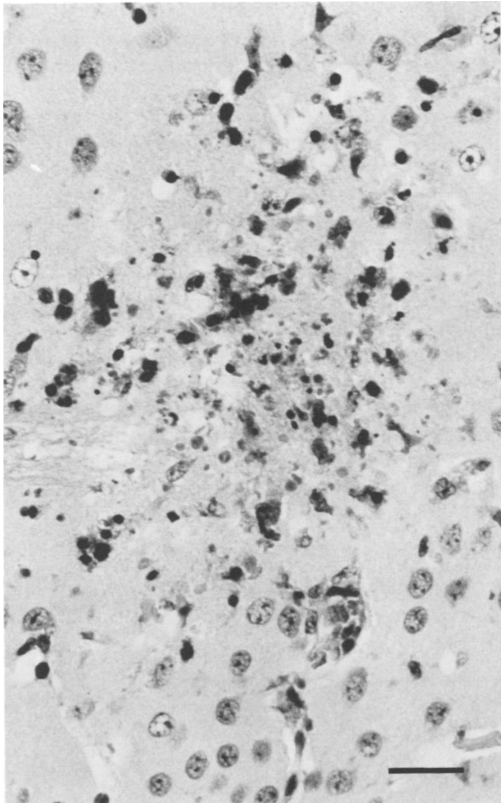

FIG. 4. *Focus of micronecrosis in neural parenchyma 7 days after infection. With H&E stain (used here), it is impossible to determine which components of the infective focus are of parasitic origin. Bar equals 30 μm.*

zoites were still present, sometimes in association with a microglial nodule, but often occurring as an isolated focus of organisms in a tiny pocket of parenchymal necrosis without significant accompanying inflammation. Very occasional groups of tiny tissue cysts (8 to 10 μm in diameter) were demonstrated by the H&E and PAP-MGP stains; these encysted forms were invariably associated with a surrounding inflammatory response. PAP-positive-staining particulate debris was also visualized in association with inflammatory cells. By day 14 the PAP stain identified more individual cells whose original cell type could not be determined and which had brown-staining antigen-antibody reaction products in their cytoplasm; again, parasitic vacuoles could not be recognized as discrete structures within these cells, nor did the cells have the appearance of typical small tissue cysts. These cells, with cytoplasmic toxoplasma antigen as well as extracellular PAP-positive debris, and rare free tachyzoites were associated with microglial nodules; focal areas of parenchymal destruction occurring in the absence of an inflammatory response now were only rarely visualized.

(ii) Intermediate infection (2.5 to 6 weeks). In the intermediate stage of infection, discrete collections of free tachyzoites were seen rarely and never in the absence of an inflammatory reaction. From 3 to 5 weeks after infection, the centers of the microglial nodules were stained heavily by the PAP stain. There were occasional discernible intact tachyzoites, but most of the positive staining was in small pieces of debris or as amorphous substance either extracellularly or in the cytoplasm of cells. After 5 weeks, the amount of antigen-antibody reaction products associated with microglial nodules decreased, but evidence of antigen remained as a component of the nodules for the duration of the study (Fig. 7). Small tissue cysts, often in clumps of three to five were frequently present at the periphery of the microglial nodules but were no longer located in the center of grouped

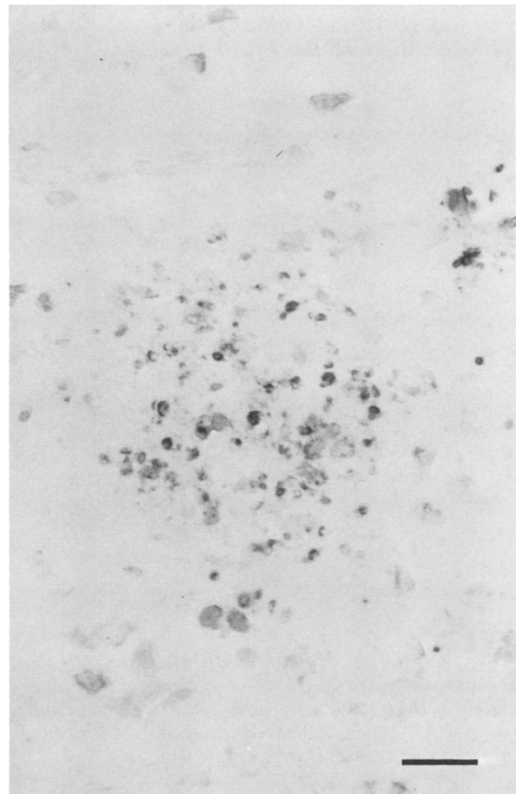

FIG. 5. *Same area as in Fig. 4. Immunohistological staining delineates tachyzoites and parasitic debris. PAP-MGP, 1:1,000 dilution of rabbit antitoxoplasma antiserum. Bar equals 30 μm.*

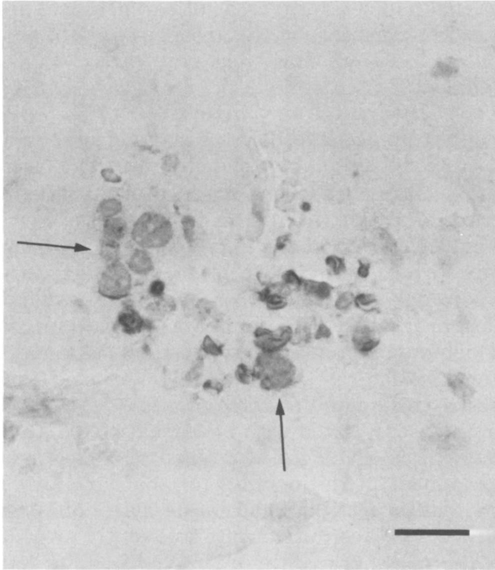

FIG. 6. *Infective focus 7 days after infection. Arrows point to cells containing cytoplasmic antigen-antibody reaction products. Free tachyzoites are also present. PAP-MGP, 1:1,000 dilution of rabbit antitoxoplasma antiserum. Bar equals 20 μm.*

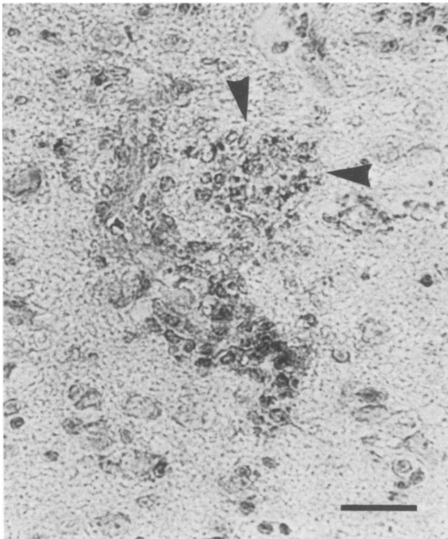

FIG. 7. *Microglial nodule 35 days after infection. Arrowheads point to an area containing tiny pieces of debris that stain positively for toxoplasma antigen(s). PAP-MGP, 1:1,000 dilution of rabbit antitoxoplasma antiserum. Bar equals 20 μm.*

inflammatory cells (Fig. 8). Cysts were also present scattered in the neural parenchyma remote from any inflammatory response or tissue disruption; many of these cysts were single. The size of the cysts increased fairly uniformly and rapidly over the first 5 weeks to an average of 30 μm. After 2 weeks of infection, there was a diffuse increase of mononuclear cellularity in the parenchyma of the brains from all infected mice that persisted throughout the entire study period. In addition, there were always focal collections of mononuclear inflammatory cells around blood vessels, near the ependyma, in the leptomeninges, or as collections of microglial nodules. The interhemispheric fissure was sometimes extensively infiltrated by inflammatory cells (Fig. 9); however, for the duration of the study, evidence of toxoplasma antigen was never seen in the leptomeninges.

(iii) Late infection (7 to 15 weeks). From 7 to 15 weeks after initial infection, the histology of the brain sections suggested that recurrent or recrudescent infection was occurring. In addition

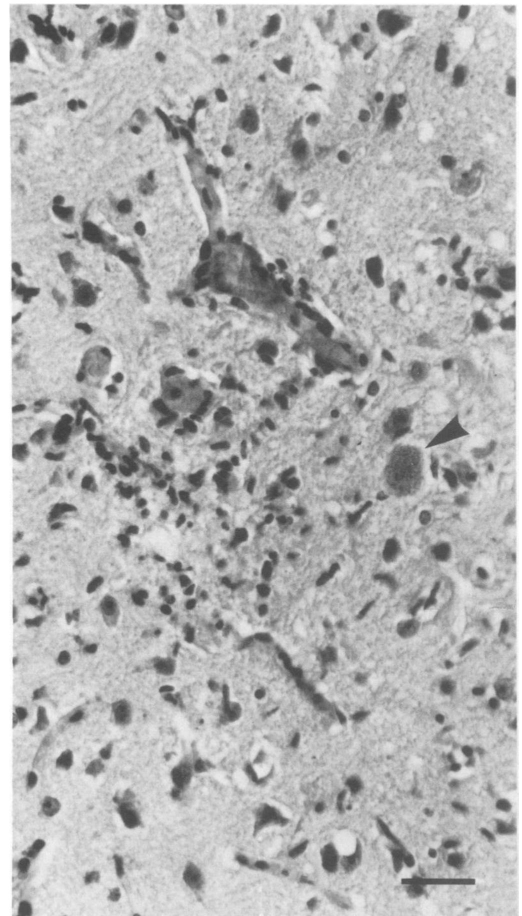

FIG. 8. *Toxoplasma tissue cyst (arrowhead) at edge of microglial nodule 42 days after infection. H&E. Bar equals 30 μm.*

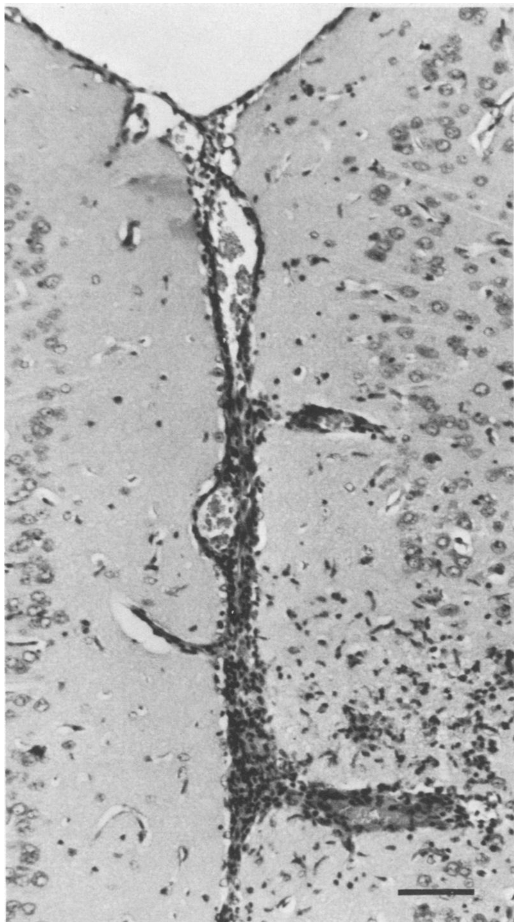

FIG. 9. *Cerebral interhemispheric fissure 58 days after infection. H&E. Bar equals 60 µm.*

to older-appearing microglial nodules containing very small amounts of antigen-antibody reaction products, fresher, more extensive neural lesions appeared containing much more toxoplasma antigen (Fig. 10). Unlike the early parasitic foci which resulted from initial infection, these recurrent foci always were accompanied by mononuclear inflammatory cells. They also showed micronecrosis and vacuolation of the involved neural parenchyma, reminiscent of the initial lesions, and contained occasional intact tachyzoites and large amounts of PAP-staining debris. Mature cysts were not observed in association with these foci. With the appearance of these new foci, brain sections contained at least two distinct populations of tissue cysts of different sizes. The smaller cysts occurred in groups and often at the periphery of an inflammatory focus (Fig. 8); the older, larger cysts were scattered randomly in the neural parenchyma apart from the smaller cysts and away from any inflammatory reaction or residual evidence of prior tissue disruption (Fig. 1). A small daughter cyst or cysts occasionally were observed attached to a large cyst; these "budded" cysts did not evoke an inflammatory response (Fig. 11).

DISCUSSION

The results of this morphological and immunohistological study of toxoplasmosis in the CNS of mice have demonstrated that toxoplasma antigen is anatomically associated with the inflammatory response in the brain parenchyma. Antigen was observed in four structural forms during toxoplasma infection: as the free tachyzoite, as particulate extracellular debris, as amorphous intracellular cytoplasmic staining, and as the tissue cyst.

During the course of infection, these structural forms of antigen were associated with a mononuclear cell inflammatory response, and it is apparent that persistent toxoplasma antigen is responsible for the formation and maintenance of the microglial nodule, one of the histological hallmarks of toxoplasma encephalitis. In the earliest stages of infection, the free tachyzoite invaded the brain in the absence of a significant inflammatory component and was capable of destroying brain cells. Neural injury may have been a prerequisite for initiating a cellular reaction in the brain, but once a reaction had been generated, our study demonstrated that, except in the leptomeninges, persistence of inflammation morphologically was associated with the

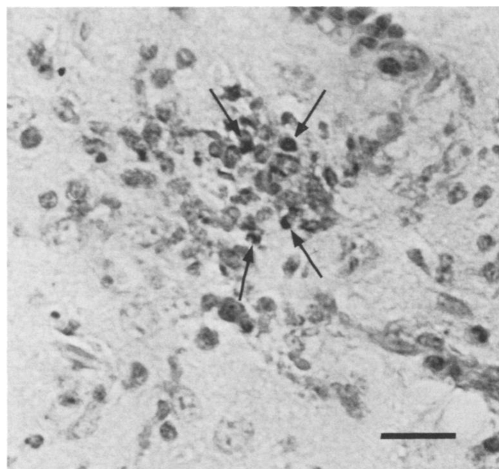

FIG. 10. *Recurrent focus of infection composed of free tachyzoites (arrows) and inflammatory cells, 49 days after primary infection. PAP-MGP, 1:10⁶ dilution of rabbit antitoxoplasma antiserum. Bar equals 20 µm.*

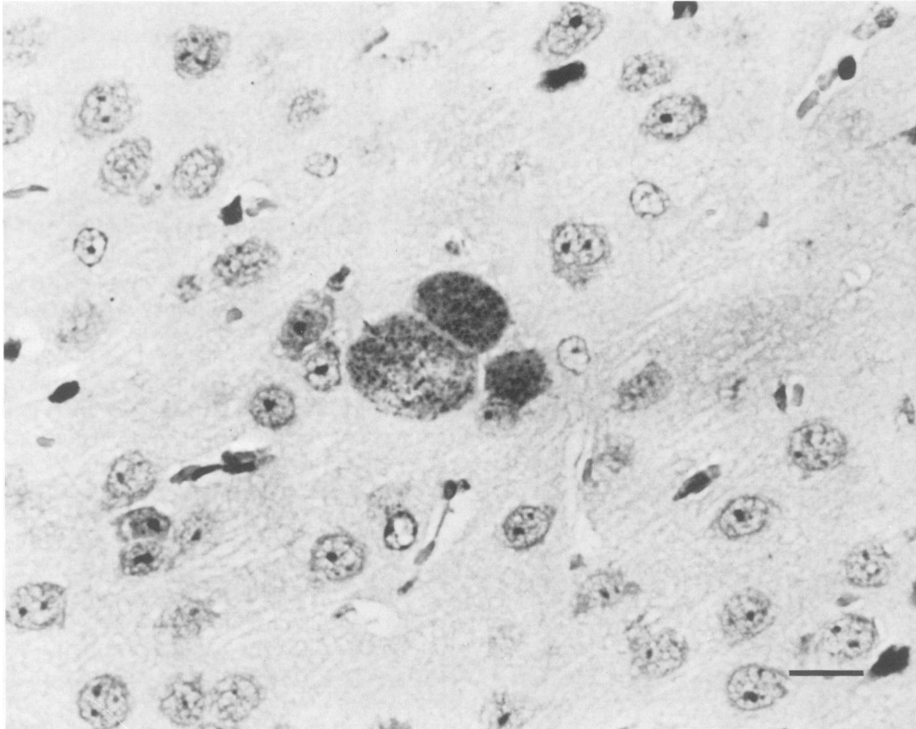

FIG. 11. *Toxoplasma tissue cyst with two daughter cysts, 76 days after infection. Note the absence of any surrounding inflammatory response. H&E. Bar equals 20 μm.*

presence of toxoplasma antigen. Once parenchymal integrity was disturbed an inflammatory response rapidly enused, the small necrotic foci of free organisms were surrounded by inflammatory cells, and a destructive process was initiated which resulted in the presence of PAP-positive-staining debris in the center of collections of mononuclear cells. Evidence of antigen remained throughout the duration of the study as amorphous PAP-positive-staining material in the center of microglial nodules. Whereas early small parasitic tissue cysts were visualized in the center of an inflammatory reaction, growing cysts did not remain in this location; cysts which survived and enlarged escaped the inflammatory cells. However, the long-term persistence of both antigen and inflammatory cells for the duration of this study indicated that cellular immune mechanisms were incapable of totally eradicating the disease process from the brains of mice.

The histological picture of chronic encephalitis in mice in this study was quite different from that found in another study (Conley et al., in press) in which the PAP method was used to study a case of acquired necrotizing toxoplasma encephalitis in an immunocompromised human patient. In the human case, and as has been reported by others (6, 9, 18), there was histological evidence that tissue cysts in the brain had ruptured, releasing concentric foci of active destructive tachyzoites. These tachyzoites rapidly infected surrounding neurons and astrocytes and could be visualized as discrete parasitic vacuoles within these infected cells. Rupture of infected cells has been related both to the virulence of the infecting strain and to the immunological status of the host (for review, see reference 20). Our mice were not immunocompromised and were infected with an avirulent strain of toxoplasma, and a mechanism involving cyst rupture and rapid infection of brain cells that would explain persistence of the infection, and thus of antigen, in the brains of our mice was not observed. Recurrent episodes of parasitemia have been suggested by morphological studies of toxoplasma cysts in the brains of mice (15, 29) and hamsters (4) and have been documented by recovery of the toxoplasma organism from the blood at time intervals (up to 8 months) after infection with avirulent strains in mice, rabbits, and guinea pigs (10, 22). A sequential study of cyst formation in the brains of mice by van der Waaij (27), in which he related the number of cysts inoculated subcutaneously to the number

of cysts subsequently recovered from the brain, provided evidence that, for each strain of toxoplasma, all cysts developed simultaneously, were fairly uniform in size, and enlarged with time. He also observed daughter cysts in close proximity to larger cysts but did not demonstrate an inflammatory reaction accompanying this budding process. His methods did not allow the accurate counting of cysts that were under 25 μm in diameter, and a second population of cysts developing in neural parenchyma apart from mature cysts may have been missed. Wanko et al. (29) studied cysts in the brains of mice by electron microscopy and found over a 60-day period that, on the basis of size, there were clearly two populations of cysts and that both large and small cysts had similar and constant fine-structural features. In agreement with Wanko et al. (29), we also observed a second population of small cysts with immunological staining characteristics which were identical to those of the large cysts. However, anatomically the small cysts were most often related to foci of inflammatory cells rather than to a well-established parental cyst, suggesting that recurrent episodes of parasitemia may be a possible etiological factor responsible for the persistence of the antigenic stimulus in the brains of our mice.

Studies of parasitemia in toxoplasma infection have documented the presence of blood-borne organisms despite concomitant high titers of circulating antibody against toxoplasma (10, 22). Cell-mediated immunity is thought to be the major mechanism of protection against toxoplasma infection (5, 21, 23, 24) and, along with antibody, may participate in maintaining the organism in its encysted form. Frenkel et al. (6) have provided convincing experimental evidence that perturbation of cellular immunity by immunosuppression results in a recrudescence of chronic infection with the development of an acute necrotizing encephalitis. By using the PAP technique, we were able accurately to follow both the free tachyzoite and the cyst forms of toxoplasma and found that, with an avirulent strain in immunocompetent mice, the encystment process was quite rapid, an observation that agrees with prior work by Huldt (12). The infection in the brains of our mice did not include a stage in which there were infected cells of diverse morphological types, each with a discrete parasitic vacuole, as had been observed in cases of acute necrotizing toxoplasma encephalitis in humans (Conley et al., in press). We also found that cysts initially formed, usually in clusters, in the middle of a focus of inflammation, suggesting that the initial encystment process was stimulated by the presence of the mononuclear inflammatory cells. As the cysts enlarged with time after infection, they tended to be located, again in clusters, at the periphery of an inflammatory focus. Later, single cysts were seen in normal neural parenchyma quite remote from, and seemingly unrelated to, any inflammatory reaction. It is difficult to account for the anatomic location of these single cysts unless there is mobility of the parasite. Lycke et al. (13), using time-lapse cinematography, demonstrated considerable in vitro mobility of tachyzoites, and Yoshizumi (31) and Ghatak and Sawyer (8) postulated that tachyzoites may move along glial and neural cell processes during the course of acquired CNS infection. Since the PAP method allowed visualization of individual organisms, we should have been able to document the spread of infection caused by mobility of tachyzoites. Instead, our observations suggested that in immunocompetent mice free tachyzoites travel little and rapidly generate a surrounding inflammatory reaction. The more probable mechanism to explain movement of a tachyzoite is that a parasite was taken up at the site of an inflammatory focus and carried into the surrounding neural parenchyma by a macrophage. "Brain macrophages" have been shown to move into and within the brain (7, 14, 17). Single cysts may be the result of such infected cells continuing to migrate until they are destroyed or rendered functionless by the growing parasitic cyst.

The formation and structure of tissue cysts has been the subject of considerable investigation (9, 15, 18, 29). Initially the cyst forms in a vacuole of cytoplasm within the infected host cell. A "limiting membrane" which is thicker in the less virulent strains of toxoplasma (15) and is of parasitic origin rapidly forms. Ghatak and Zimmerman (9) and Wanko et al. (29) thought that the ultimate wall of the mature cyst represented an interaction between components derived from the host cell wall and those manufactured by the parasite, and immunological studies have revealed that the surface of the mature cyst contains an antigen(s) of parasitic origin (1, 12, 16). With light microscopy and the PAP method, we also could demonstrate immunological staining of the cyst wall. However, since cysts are found away from any inflammatory response, it is obvious that the toxoplasma antigen(s) of the cyst wall is in itself not sufficient to elicit a cellular reaction. It is possible that if the cyst wall is composed of combined elements of host cell and parasitic origin, the inclusion of components from the host cell is sufficient to prevent a cyst from being recognized as "foreign" by the infected animal and appropriate protective immunological mechanisms (i.e., those which

would lead to destruction of the cyst) would not be activated.

Chronic infection of the CNS with toxoplasma appears to be the result of a balance between the ability of the host to destroy extracellular organisms before they are able to infect significant numbers of neuronal or glial cells and the ability of the parasite to gain rapid access to, and multiply in, an immunologically protected intracellular environment. The constant presence of a population of mononuclear cells appears to be essential in maintaining this balance.

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