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Pathological changes in the brains of mice infected with *Toxoplasma gondii*: a histological, immunocytochemical and ultrastructural study

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Summary. The pathological changes in the brains of mice infected with *T. gondii* were studied at various intervals between 7 days and 22 months post-infection using histology, immunocytochemistry and electron microscopy. Initially, a few single parasites were observed (day 7) but necrotic lesions and microglial and inflammatory nodules rapidly appeared (9–14 days). The majority of the lesions between days 9 and 14 contained proliferating toxoplasms and early cyst formation but from 21 days onwards the vast majority of nodules contained neither parasites nor *Toxoplasma* antigen. Intact intracellular cysts persisted throughout the period of study eliciting no host response. A generalized meningoencephalitis developed by day 11 and persisted with varying degrees of severity throughout the 22 months studied. At first, the inflammatory cells consisted of lymphocytes and monocyte/macrophages but during the chronic phase plasma cells predominated. In chronic infections, the number of microglial/inflammatory nodules was relatively constant with only a few containing toxoplasmic material resulting from recent cyst rupture. A few brains contained small nodules of dystrophic calcification. This study shows that in these asymptomatic animals, the major feature is perivascular cuffing by mononuclear cells and localized microglial/inflammatory nodules. After the development of the chronic state, there is no obvious increase or decrease in the severity of the pathological changes with time.

Keywords: *Toxoplasma gondii*, brain, pathology, immunocytochemistry, ultrastructure

Infection with *Toxoplasma gondii* results in an initial acute phase with rapid multiplication and dissemination of the parasite throughout the body. With the onset of the host immune response, the infection enters the chronic phase with the parasite limited to tissue cysts predominantly located in the central nervous system, striated and heart muscle (Jacobs, 1967). Viable parasites within these cysts persist for long periods probably throughout the life of the host (Tadros & Laarman 1982). In the majority of cases, chronic infections of immunocompetent animals produce no clinical symptoms.

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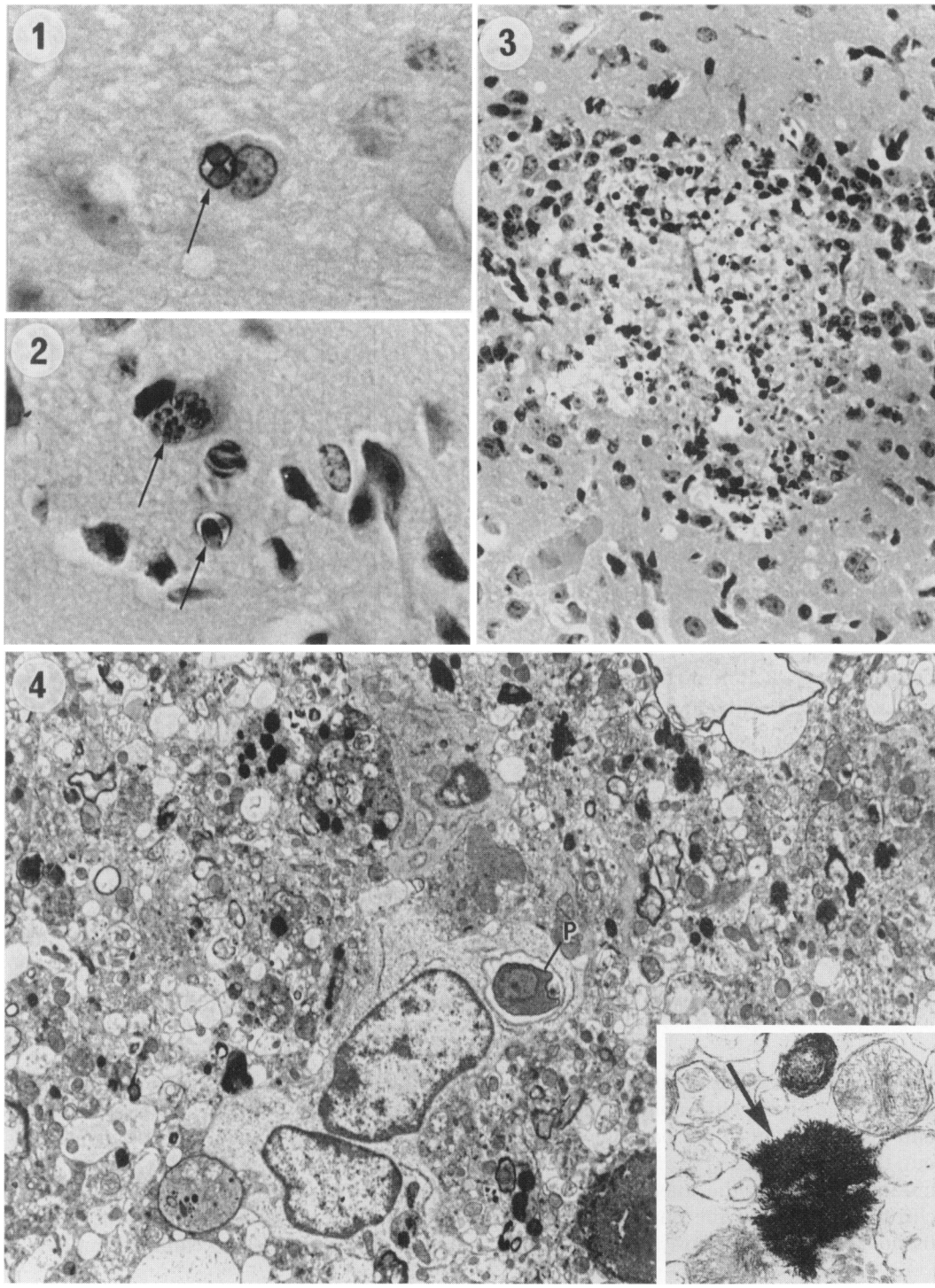
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However, it has been shown that factors which result in immunosuppression (chemotherapy, organ transplantation and acquired immune deficiency syndrome) can result in serious neurological disease due to the recrudescence of actively proliferating toxoplasms (Frenkel *et al*, 1975; Navia *et al*, 1986). There have been a number of studies of the pathological changes in the brain associated with acute and early chronic (1–3 months) infection (Frenkel 1956; Graham *et al*, 1984; Kittas *et al*, 1984; Beverley & Henry 1971; Conley & Jenkins 1981) with one study extended to 9 months post-infection (Ito & Tsunoda 1968). In the present study, we have extended the period of investigation to 22 months post-infection (PI), which is closer to the normal life-span of the mouse, to examine any changes in the pathological lesions with time. In addition, we have used immunocytochemistry and electron microscopy to provide more detailed information on the structure of the lesions and their relationship to the presence of toxoplasms.

Materials and methods

In the present study, an outbred (STR) strain of mice and the avirulent SRA strain of *T. gondii* were employed (Hutchison 1986). A total of 65 mice were inoculated subcutaneously with tissue of *T. gondii* obtained from the brains of mice infected 2 months previously. Animals were examined at 7 (4 mice), 9(4), 11(5), 12(6), 14(2), and 21(4) days after inoculation of approximately 100 tissue cysts and at 1(4), 1.5(5), 6(8) 12,(12), 15–17(5) and 18–22(6) months after inoculation of approximately 12 tissue cysts. Six uninfected mice were included as controls.

To exclude the possibility of fixation artifacts at the ultrastructural level, eight of the mice (3 at 1.5 months, 3 at 6 months and 2 at 12 months) were fixed by whole body perfusion with 3% glutaraldehyde. With the other 57 mice, the brain was removed immediately after the animal was killed and divided longitudinally into equal halves. One half was placed directly into 3% glutaraldehyde and chopped into 1 mm cubes. Blocks from the perfusion and immersion fixed material were processed for electron microscopy as described previously (Ferguson & Hutchison 1987a, b). Cysts and lesions were identified in 1 μm Azure A stained sections prior to ultra-thin sectioning for electron microscopy. The other half of the brain was fixed in phosphate buffered formalin (Carson *et al*, 1973), dehydrated, and embedded in wax. For histological examination, longitudinal sections through the brains were stained with haematoxylin and eosin.

Immunocytochemistry

A standard two-stage indirect immunocytochemical technique was applied using a polyclonal anti-*Toxoplasma* antibody with diaminobenzidine as the chromogen. To compare mice at different time intervals post infection, the numbers of organisms, lesions and cysts were quantified in two longitudinal sections through the brain of each mouse.

Results

At 7 days post infection (PI), the brains were histologically normal and similar in appearance to the uninfected controls. However, by immunocytochemistry it was possible to

Fig. 1. Light micrograph of an immunostained section showing two parasites within a neurone (arrow). Note the positive staining of both the parasites and the parasitophorous vacuole membrane. 7 day infection. × 2000. **Fig. 2.** Immunostained section showing a group of proliferating parasites (arrows) within normal appearing neuropil. 9 day infection. × 1500. **Fig. 3.** Light micrograph of a necrotic lesion, with pyknotic nuclei, disrupting the normal architecture. 9 day infection. H&E stained. × 400. **Fig. 4.** Electron micrograph of part of a necrotic lesion showing the large areas of debris. Note the intact microglial-like cell containing a proliferative form of the parasite (P). 9 day infection. × 5500. Insert. Enlargement from a necrotic lesion showing early calcification (arrow). × 40 000.

Table 1. Average number of organisms, lesions and cysts per mouse at various time intervals post-infection*

Period PI	Mice (no.)	Organism (no. of foci)	Necrotic lesions	Nodules +ve Tox	Nodules −ve Tox	Cysts (no.)
7 days	4	0.75 (0–2)†	—	—	—	—
9 days	4	5 (2–7)	0.5 (0–1)	—	—	—
11–14 days	13	1.7 (0–5)	2.2 (0–7)	6.9 (1–16)	3.0 (0–14)	2.6 (0–6)
21 days	4	—	—	3.75 (1–4)	19 (16–23)	6 (3–9)
1–22 months	32	—	—	0.16 (0–1)	3.9 (1–8)	8.44 (1–14)

*Based on the quantitation of two immunocytochemical stained sections through each brain.

† Figures in parentheses represent the range observed.

identify a few individual intracellular parasites within the neuropil in certain mice (Fig. 1). The majority of such parasites were observed in the olfactory lobes and cerebrum. Both the parasite and the wall of the parasitophorous vacuole stained positively with the polyclonal anti-*Toxoplasma* IgG (Fig. 1). This was the only evidence of parasite involvement in the brain (Table 1).

By 9 days, a few small groups (6–12 organisms) of intra- and extra-cellular parasites were observed (Fig. 2). Such groups and single parasites with no associated host reaction were also observed, but less frequently, in mice at 11 and 12 days PI (Table 1).

In addition, a number of the mice exhibited necrotic lesions which appeared as lucent, vacuolated areas of variable size with pyknotic nuclei in H&E stained sections (Fig. 3). By immunocytochemistry the lesions showed a weak diffuse reaction with a few strongly stained toxoplasms predominantly towards the periphery of the lesions. Ultrastructurally, it was observed that the lesion consisted of cell debris from a general lysis of both neural and glial cells (Fig. 4). In certain cases, numerous clumps of small crystals consistent with early calcification were observed among the cell debris (Fig. 4 insert). Only rare intact microglial or inflammatory cells were observed within the lesion (Fig. 4). The few organisms present had the ultrastructural features of endozoites. This type of lesion was also observed in greater numbers at 11–14 days PI but not thereafter (Table 1).

Between 11 and 14 days PI, the most common observation was the presence of microglial or inflammatory nodules (Table 1). These microglial nodules were normally small, consisting of collections of microglial cells plus a few disrupted host cells with no general host cell lysis (Fig. 5). The inflammatory nodules were larger and consisted of numerous macrophages and, in certain cases, polymorphonuclear neutrophils were also present (similar to Fig. 6). There was evidence of some cell death by apoptosis within these lesions (Fig. 8). Numerous proliferating toxoplasms were observed within the majority of lesions (Fig. 5). By electron microscopy, they were identified

within both neural and inflammatory cells and the host/parasite relationship was similar to that of the proliferative form (see Ferguson & Hutchison 1987b for details). Small cysts were also identified by immunocytochemistry and electron microscopy in areas of normal appearing neural tissue (Fig. 7).

During this period (11–14 days PI), a meningoencephalitis developed. There also appeared to be some sequestration of leucocytes within the blood vessels of the neuropil (Figs 7 and 10). Lymphocytes and monocytes were observed traversing the endothelium (Fig. 11) resulting in numerous inflammatory cells cuffing the blood vessels. No parasites or *Toxoplasma* antigens were associated with these inflammatory changes.

At 21 days PI, a number of larger microglial/inflammatory nodules were observed (Fig. 6). At this time, only a few proliferative organisms could be identified by either immunocytochemistry or electron microscopy (Fig. 8), although a number of small cysts were present. In those lesions containing toxoplasms, there was a change in the host/parasite relationship compared to days 11–14. The parasites within the macrophages showed evidence of degradation rather than proliferation (Fig. 9). The majority of nodules (84%) were immunologically negative (Table 1). Ultrastructurally, the macrophages in these nodules contained large heterophagosomes with unrecognizable cell debris (Fig. 8). The majority of developing tissue cysts were in areas of the neuropil not associated with any lesion. The generalized inflammatory changes were similar to those described at 11–14 days PI (Figs 7 and 10).

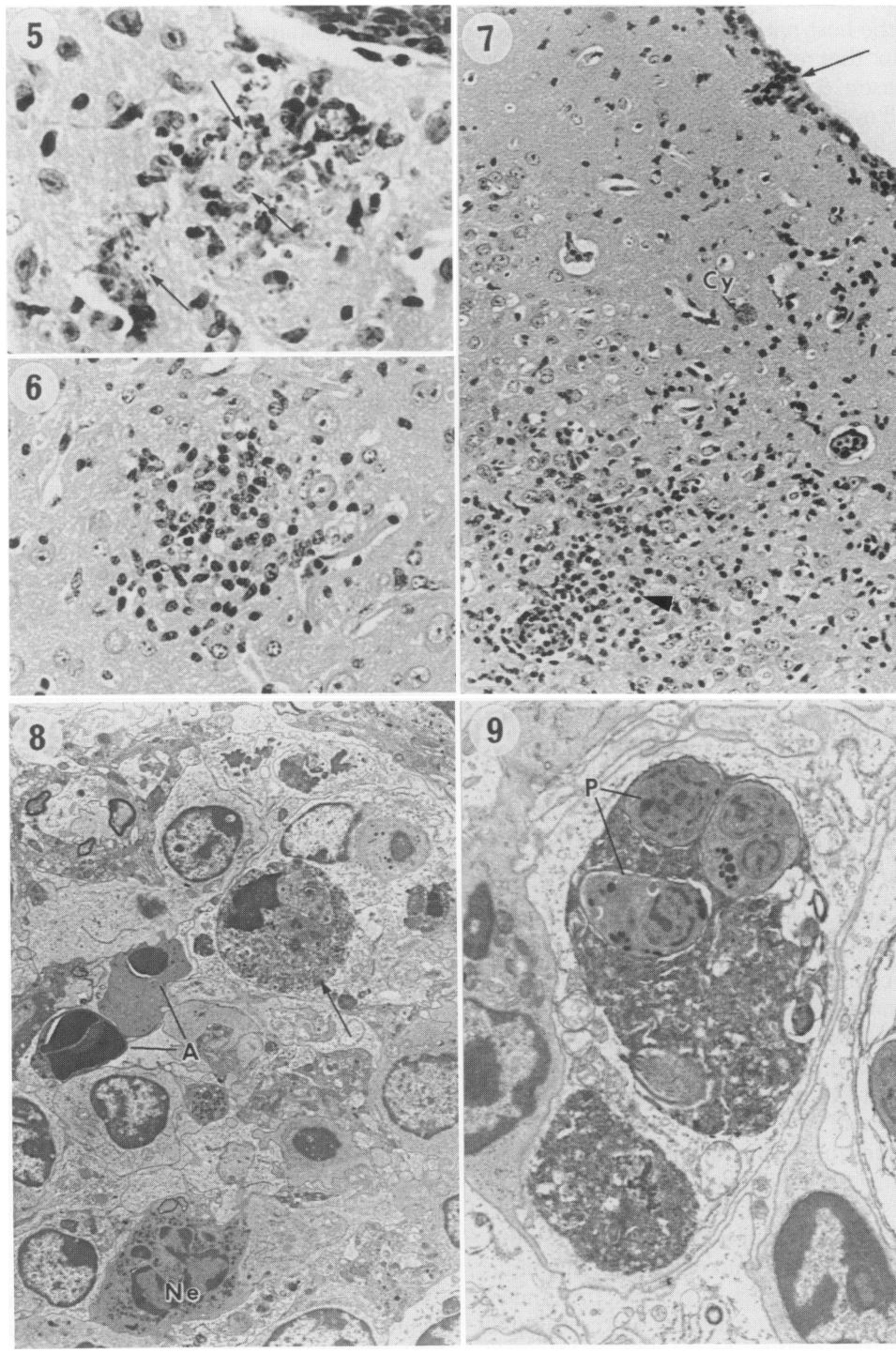
In the chronically infected mice, although there were individual variations, similar results were observed irrespective of the interval post infection from 1 to 22 months. Small numbers of microglial and inflammatory nodules, which disrupted the underlying brain architecture, were present in all brains (Fig. 15) but there was no evidence of increasing numbers with period post infection. A very small proportion (3%) of the nodules contained immunoreactive material (Table 1) or extracystic toxoplasms. These appeared to arise from the recent rupture of a tissue cyst associated with a rapid influx of inflammatory cells (see Ferguson *et al.* 1989 for details). There was little evidence of parasite proliferation or new cyst formation associated with these lesions. The majority of lesions contained no immunoreactive material and consisted of groups of macrophages admixed with a few plasma cells and lymphocytes (Fig. 15). The macrophages contained numerous debris containing phagosomes or lipid droplets (Fig. 16). Throughout the chronic phase, individual and small groups (2–6) of tissue cysts were observed unassociated with microglial/inflammatory nodules (Fig. 12). Ultrastructural examination confirmed the intracellular location of all the tissue cysts throughout the period studied (see Ferguson & Hutchison 1987a, b; Ferguson 1988).

Varying degrees of inflammation were present throughout the 22 months studied. For example, large numbers of inflammatory cells, predominantly plasma cells, with a few lymphocytes and monocytes/macrophages, were found around the blood vessels and underlying the meninges (Figs 12–14). A number of plasma cells were also distributed throughout the neuropil. No parasites or *Toxoplasma* antigens were associated with these changes.

In addition, small areas of dystrophic calcification were observed in only a few of the chronically infected mice (4 out of 32) (Fig. 17). One mouse was found to have a large zone of calcification. The incidence of dystrophic calcification was not affected by the period post infection. There was no identifiable lesion or reaction to the areas of calcification.

Discussion

The appearance of parasites and the subsequent development of lesions in the brain is basically similar to that described previously (Conley & Jenkins 1981; Kittas *et al*, 1984;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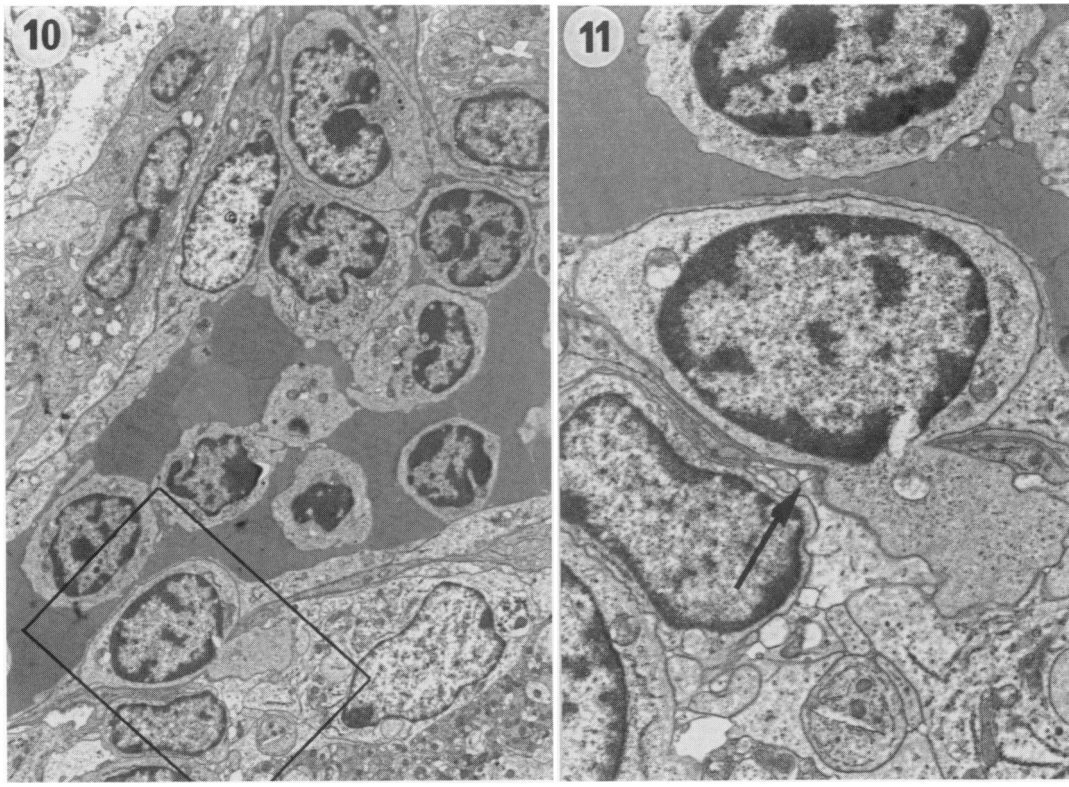

Fig. 10. Low power electron micrograph of a blood vessel showing a number of monocytes and lymphocytes sequestered within the lumen and also around the outside of the vessel. 21 day infection. × 3300. **Fig. 11.** Detail of the enclosed area in Fig. 10 showing a lymphocyte traversing the endothelial lining of the vessel (arrow). × 10 000.

Ito & Tsunoda 1968). The slight variations in time could be due to the methods of infection or the strain of *Toxoplasma* or mouse used. This study also confirms the advantages of immunocytochemistry in following the early stages of colonization (Conley & Jenkins 1981; Kittas *et al*, 1984). The large inoculum of tissue cysts was used, in the present study of the acute phase, to increase the incidence of lesions in the brain making ultrastructural examination feasible. There is no evidence that this inoculum resulted in the appearance of lesions not normally associated with the acute phase (Conley & Jenkins 1981).

Initial colonization during the acute phase is characterized by the development of two

Fig. 5. Light micrograph of an early microglial nodule containing numerous proliferating toxoplasms (arrows). 12 day infection. H&E stained. × 1100. **Fig. 6.** Nodule consisting of numerous inflammatory cells with no visible parasites. 21 day infection. H&E stained. × 700. **Fig. 7.** Light micrograph of part of the cerebrum in which an inflammatory nodule (arrowhead) and infiltrate of the meninges is shown (arrow). Note the small tissue cyst (Cy) within the normal neuropil. 21 day infection. H&E stained. × 225. **Fig. 8.** Electron micrograph of an inflammatory nodule consisting of macrophages containing cell debris (arrow) and a polymorph neutrophil (Ne) plus a few apoptotic bodies (A). 21 day infection × 2700. **Fig. 9.** Detail of part of an inflammatory nodule showing a number of parasites (P) within the phagolysosome of a macrophage admixed with cell debris. 21 day infection × 5400.

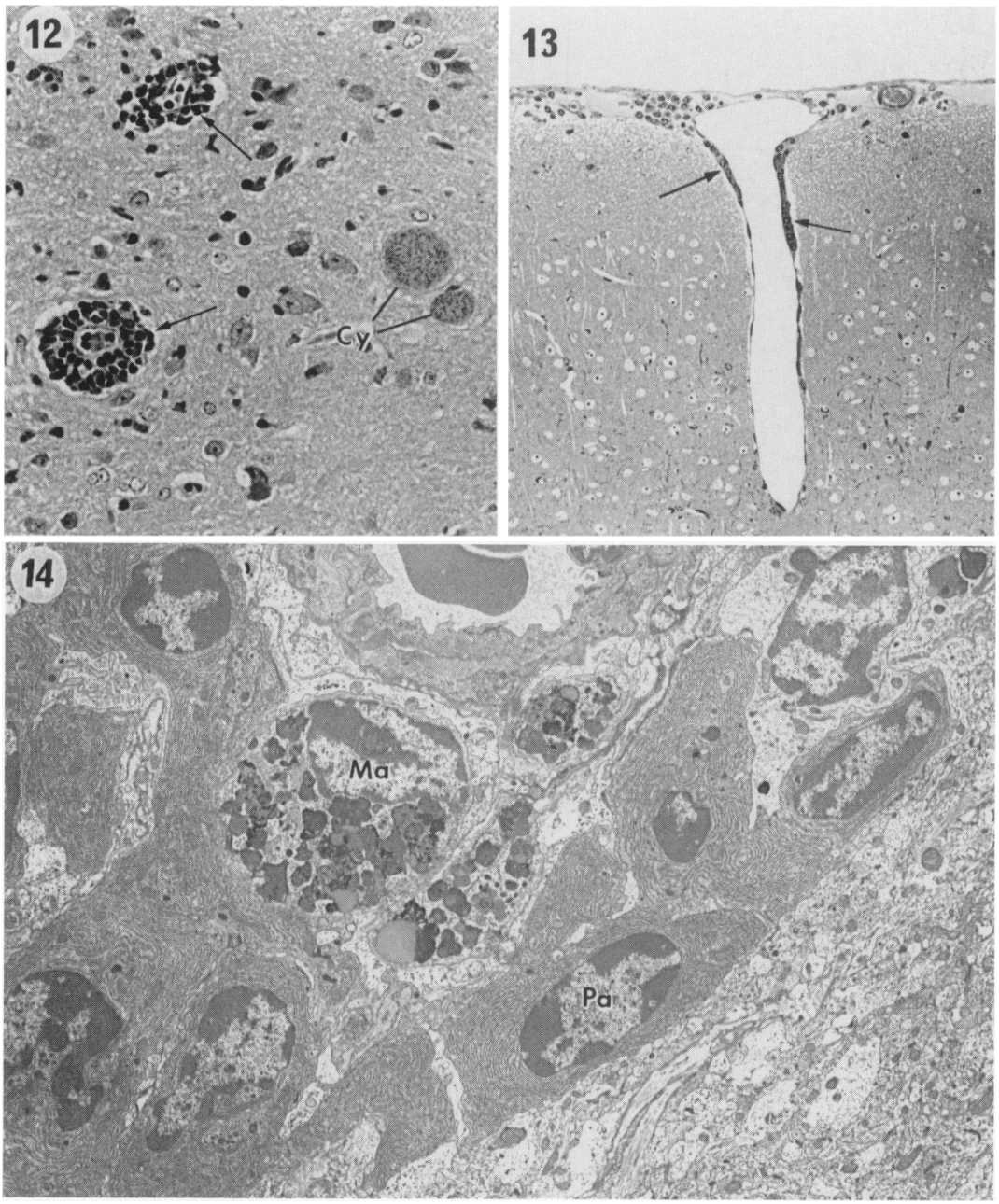

Fig. 12. Light micrograph showing numerous inflammatory cells cuffing two blood vessels (arrows). Note the absence of any reaction to the tissue cysts (Cy). 12 months infection. H&E stain. × 800. **Fig. 13.** Light micrograph of the periphery of the cerebrum showing a number of inflammatory cells within the meninges and also cuffing the blood vessel (arrows). 6 months infection. Perfusion fixed, plastic embedded. Azure A stained. × 400. **Fig. 14.** Electron micrograph illustrating the numerous plasma cells (Pa) and macrophages containing cell debris (Ma) cuffing a blood vessel. 18 months infection. × 5000.

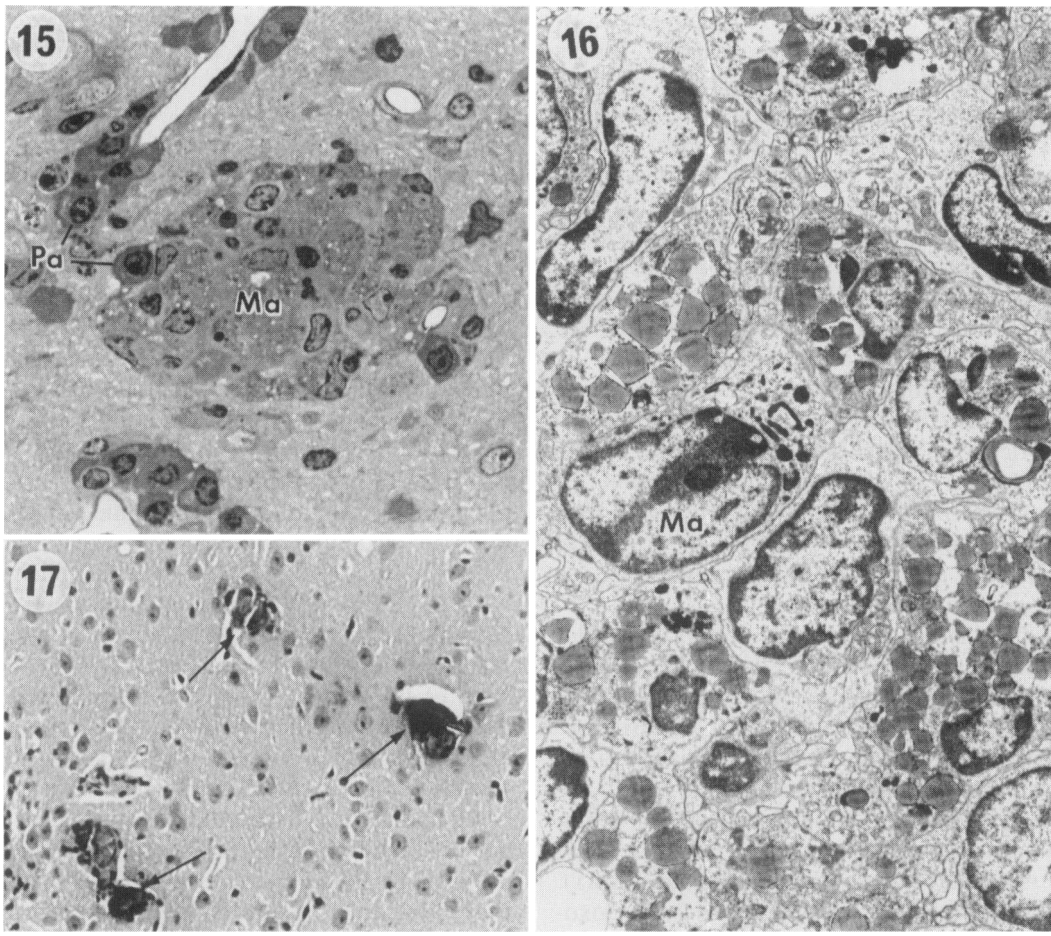

Fig. 15. Light micrograph of an inflammatory nodule from a chronic infection showing it to consist of numerous macrophages (Ma) and a few plasma cells (Pa). 12 months infections. Perfusion fixed, plastic embedded. Azure A stained. × 1000. **Fig. 16.** Electron micrograph of part of an inflammatory nodule similar to that in Fig. 15 showing a number of macrophages (Ma) containing numerous lipid droplets. 12 months infection × 6000. **Fig. 17.** Light micrograph showing a number of area of dystrophic calcification (arrows). 18 months infection. H&E stained. × 400.

types of lesions; the necrotic lesion and the small microglial or inflammatory nodule. The relatively large necrotic lesions are limited to the early phase of infection (9–14 days) and are similar to the lacy lesions described by Conley and Jenkins (1981). These lesions differ from the early inflammatory nodules by the large area of cell necrosis with relatively few parasites or inflammatory cells present. This was confirmed by both immunocytochemistry and electron microscopy. The reason for this extensive cell necrosis is unclear but possible factors could be the release of (1) a toxin by the parasites; (2) lymphokines by inflammatory cells or (3) it may represent small infarcts due to localized blood vessel occlusion at sites of parasite invasion. However, the first two possibilities are inconsistent with the observation that microglial/inflammatory nodules which contain large numbers of parasites and inflammatory cells are not associated with

widespread cell necrosis. Nor could we confirm the presence of occluded vessels although vessels containing groups of platelets without fibrin deposition were observed. The ultrastructural analysis also showed evidence of early stages in the calcification of the cell debris in certain lesions. It is therefore possible that the rare dystrophic calcification observed in chronic infection develops from these lesions formed during the acute phase. This is consistent with the lack of evidence for calcification associated with cysts or microglial/inflammatory nodules where cell debris is efficiently removed by the phagocytic cells. This may explain the higher incidence of dystrophic calcification in intrauterine infections (Graham *et al*, 1984) where there will be greater neurological damage and fewer inflammatory cells to remove the debris.

The microglial or inflammatory nodules which start to appear by day 11 are present throughout the acute and chronic phases of infection. These lesions vary in structure with the phase of infection. They are usually smaller than the necrotic lesions and in the early stages contain large numbers of proliferating toxoplasms and relatively few inflammatory cells (Conley & Jenkins 1981). Ultrastructural studies have shown the toxoplasms proliferate within both neural and inflammatory cells (Ferguson & Hutchison 1987b). However, this relationship rapidly changes and by day 21, the majority of nodules contain few toxoplasms. The rapid disappearance of immunostaining from the nodules differs from that reported previously (Conley & Jenkins 1981) but this may be related to the use of different polyclonal antibodies or the more efficient degradation of the *Toxoplasma* antigens in the present study. In the few cases where toxoplasms were observed they were present in phagolysosomes of macrophages with other cell debris and were undergoing degradation rather than proliferation. This change in the host/parasite relationship is similar to that seen during cyst rupture in chronic infection (Ferguson *et al*, 1989) and differs from that seen during the proliferative phase (Ferguson & Hutchison 1987b). The changes in the host/parasite relationship within macrophages between days 11 and 21 would be consistent with the activation of macrophages associated with the development of the cell mediated immune response. It is probable that the initial inflammatory infiltration is a nonspecific response to tissue damage and is associated with the presence of both macrophages and neutrophils while the latter lesions consist of activated macrophages capable of eliminating the proliferative form of the parasite (Remington *et al*, 1972).

During the chronic phase, the microglial/inflammatory nodules showed some variations in structure and activity. The vast majority consisted of macrophages filled with cell debris with no identifiable *Toxoplasma* antigen or parasites, admixed with a few lymphocytes and plasma cells. The presence of plasma cells was not observed in lesions during the acute phase. A few active lesions did contain antigens and/or parasites and these appeared to be related to recent cyst rupture (Ferguson *et al*, 1989). These observations would be consistent with the proposal by Frenkel and Escajadillo (1987) that the nodules in chronic infections represent 'tomb stones' to ruptured cysts. With the efficient immune response, neurological damage is limited to relatively small lesions which, in small numbers, are unlikely to result in any clinical symptoms. This would explain the benign nature of acquired *Toxoplasma* infections. However, these lesions could have a cumulative effect which, in large numbers, will result in clinical symptoms. This could be consistent with the reported clinical symptoms for the Panamanian night monkey (*Aotus lemurinus*) (Frenkel & Escajadillo 1987) and in congenitally infected mice (Graham *et al*. 1984).

It has been reported that there are progressive pathological changes during the first 6 weeks of infection (Kittas *et al*, 1984). It was not possible to evaluate quantitative differences in the present experiments because of

the different inoculum used to study the acute and chronic phases. However, quantitation of the lesions of similarly infected mice during the chronic phase (1–22 months) showed no difference with time. The continuing presence of nodules and inflammatory changes is similar to that reported for up to 8 months (Ito & Tsunoda 1968). In the present study of the mouse model, we found little evidence for the involvement of the ventricles in the lesions which is similar to that reported by others (Conley & Jenkins 1981; Ito & Tsunoda 1968; Graham *et al*, 1984) but differs from that of Kittas *et al.* (1984).

The development of meningitis and perivascular cuffing during the acute phase and its persistence during the chronic phase is similar to that reported in previous studies of the mouse model (Ito & Tsunoda 1968; Conley & Jenkins 1981; Kittas *et al*, 1984). The attachment of leucocytes to the endothelial cells of the cerebral blood vessels and their passage into the perivascular spaces is similar to that reported for experimental autoimmune encephalomyelitis (Raine *et al*, 1990) although there is no evidence of demyelination in the present study. The reason for the perivascular cuffing is unclear but it may represent an upregulation of endothelial cell receptors for leucocytes such as the intercellular adhesion molecules (ICAMs) (Stoolman 1989). It is possible that the breaching of the blood/brain barrier by the parasite or the subsequent neurological damage initiates this reaction. Unfortunately, no frozen material or suitable antibodies were available to study the changes in ICAMs by immunocytochemistry. It was shown that there were no apparent parasitic antigens material associated with the perivascular cuffing (Conley & Jenkins 1981; Kittas *et al*, 1984). The change from acute to chronic phases was associated with a change from a lymphocyte/monocyte population to one with a predominance of plasma cells. Another feature not reported previously was the distribution of individual plasma cells within the neuropil. It was found that although the severity of the meningitis varied between chronically infected animals, all animals showed some cuffing of the blood vessels. It is possible that this variation in the inflammation may represent the effects of intermittent cyst rupture with re-stimulation of the immune system. However, this persistent inflammation of the brain in the mouse model differs from observations in asymptomatic chronic human infection where perivascular cuffing is not a constant feature (reviewed by Scaravilli 1984). This may be due to the susceptibility of the mouse which results in a high tissue cyst density compared to the human where cysts are extremely rare. Thus, although cyst rupture is extremely rare in the mouse (Ferguson *et al*, 1989), the large number present probably means that random cyst rupture will occur more frequently in the mouse than man. This feature may be responsible for the persistent inflammation in the mouse. Thus, although the mouse is an excellent model for producing chronic infections, it may be a poor model of the neurological features of chronic human infections.

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