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A histological and immunohistochemical study of the changes induced in the brains of white mice by infection with *Toxoplasma gondii*

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Summary. The topography and the severity of the brain lesions induced in male and female white mice 1–6 weeks following inoculation with *Toxoplasma gondii* were studied by conventional histological and peroxidase–antiperoxidase (PAP) immunohistochemical techniques. The medulla oblongata and fourth ventricle were the most severely damaged areas of the brain, while the cerebellum was only minimally involved and this only in the later stages of the disease. *Toxoplasma* cysts were revealed by the PAP method as early as 2 weeks after infection. The number of cysts and the severity of the lesions increased gradually each week following inoculation. The lymphoplasmacytic element in the lesions was less intense in male than in female mice. The significance of these findings in respect to the possible immunological pathogenesis of CNS toxoplasmic changes is discussed. A correlation of the experimental findings with the known neurologic manifestations of the disease is also attempted. The advantage of the PAP method in the study of toxoplasmic infection is stressed.

Key words: toxoplasmosis, CNS, histology, topography, immunohistochemistry

Central nervous system (CNS) involvement by acquired toxoplasmosis is by no means rare (Remington & Cavanaugh 1965), especially in patients with malignancy and immunosuppressive therapy (Townsend *et al.* 1975). Neurologic manifestations of the disease are variable and reflect the presence of diffuse meningoencephalitis (Hafström 1959), a space-occupying mass (Townsend *et al.* 1975), obstructive hydrocephaly (Best & Finlayson 1979) or other forms of CNS lesions (Greenlee *et al.* 1975).

A histological study of human CNS changes at various stages of acquired toxoplasmosis is not possible and knowledge of the histopathology of the disease is based mainly on autopsy findings (Ruskin & Remington 1976; Best & Finlayson 1979). In order to elucidate the variability of the clinical manifestations of toxoplasmic encephalopathy, an experiment was performed to study the sequential changes induced at various time intervals by toxoplasmic infection in the brains of mature white male and female mice. In addition to conventional staining techniques, the peroxidase–antiperoxidase (PAP) immunohistochemical method was also employed.

Materials and methods

Thirty-five 10-week-old mature white male

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and 35 female mice of a non-inbred laboratory strain were used. The mean weight of the males was 39.5 g and of the females 29.75 g.

Thirty male and 30 female mice were inoculated with a low-virulence, cyst-forming strain of *Toxoplasma gondii* (Beverley strain). The inoculum consisted of 30 *Toxoplasma* cysts harvested from mouse brains 9 weeks after infection and given as a dilute emulsion subcutaneously. The remaining five male and five female mice were used as controls. Five infected mice of each sex were anaesthetized and exsanguinated via the axillary vessels 1, 2, 3, 4, 5 and 6 weeks following inoculation. Coronal sections of the brains were taken through four identification landmarks and contained the following structures: *Section a*: piriform area, external capsule, caudate nucleus, anterior part of hippocampus, insula collejae and septum; *Section b*: parietal lobe cortex, lateral ventricles, claustrum, amygdaloid nucleus, olfactory nodule, cortico-subthalamic tract, anterior commissure and nucleus brocae; *Section c*: occipital lobe cortex, red nucleus, nuclei of Darkschweitsch and Forel, superior calliculi, medial geniculate bodies, Edinger-Westphal nucleus and aqueductus; and *Section d*: fourth ventricle, medulla oblongata, cerebellum, nucleus ambiguus and spinal vestibular nucleus. These four coronal slices of brain tissue, each 2 mm in width, were fixed in 10% formalin, processed to paraffin, cut at 5 μm and finally stained with haematoxylin and eosin and Giemsa stains. The peroxidase–antiperoxidase (PAP) immunohistochemical technique was also used for specific demonstration of *Toxoplasma gondii* cysts, since these structures are easily missed with conventional histological stains.

Specific antiserum to *Toxoplasma gondii* was obtained from rabbits inoculated with a cyst-forming, low-virulence strain of *Toxoplasma gondii* (Beverley strain). The rabbit anti-*Toxoplasma* antiserum had a Sabin-Feldman dye-test titre of 1:2048. Dye-test negative serum was obtained from normal uninfected rabbits. Normal swine serum, swine anti-rabbit serum and the PAP complex were obtained from Dakopatts (Copenhagen, Denmark).

Immunohistochemical investigations were performed on unstained deparaffinized and hydrated 5-μm sections, using the following modified PAP method of Sternberger *et al.* (1970).

Endogenous peroxidase activity was blocked by treating the sections for 30 min with methanol solution containing 0.3% H_2O_2. Non-specific background staining was reduced with normal swine serum. The sections were incubated with the specific *Toxoplasma gondii* rabbit antiserum at a dilution of 1:40 at room temperature in a moist chamber for 30 min. Swine anti-rabbit serum and the PAP complex were subsequently applied at dilutions of 1:40 and 1:60 respectively. Colour reaction was obtained by incubation of the sections in a freshly prepared diaminobenzidine (DAB) solution (6 mg DAB with 0·01% H_2O_2 in 10 ml of tris buffer).

All sections were counterstained with haematoxylin. The sections were thoroughly washed between stages in tris saline (1/10 dilution of tris buffer, pH 7.6, in normal saline). Substitution of non-immune, dye test-negative, rabbit serum for the *Toxoplasma gondii*-specific antiserum and a slide treated with DAB alone served as controls.

The severity of the lesions in histological sections was semiquantitatively assessed on a (−) to (+++++) sign basis as previously reported (Kittas & Henry 1979). Sign (−) indicated no histological alteration, while signs (+) to (+++++) were indicative of the progressive severity of the lesions. The sum of the signs for each of the five animals in a group is the group score.

The part of the brain which was not processed for histological and immunohistochemical examination, was emulsified with isotonic sterile saline and examined for *Toxoplasma* cysts as a wet, unstained film. The total number of cysts for that particular—and constant—part of the brain was estimated (Kittas & Henry 1980). The differ-

ences in the number of cysts between the various groups were analysed statistically by Student's *t*-test.

Antibody tests in all infected mice were performed by a modification of the dye test of Sabin & Feldman (1948).

Results

The mean number of *Toxoplasma* cysts per group, found in the constant part of the brain, are shown in Table 1. There is a significant and gradual weekly increase in the number of cysts in brains of infected mice of both sexes.

The PAP method produced a moderate to strongly positive staining reaction for *Toxoplasma* cysts in the brain tissue sections of all infected mice, except those killed 1 week following inoculation. Sections from infected brains processed by the PAP technique with substitution of non-immune, dye test-negative rabbit serum, for the *Toxoplasma gondii*-specific antiserum gave negative results.

The PAP technique was superior to routine histological stains, which often failed to demonstrate *Toxoplasma* cysts in the following cases: (a) in early stages of the disease (Fig. 1), (b) in cases of cysts situated close to the Purkinje cells of cerebellar cortex or close to clusters of nerve cells of brain nuclei (Fig. 2) and (c) in heavily infiltrated and necrotic brain areas (Fig. 3).

Table 1. Mean numbers of *Toxoplasma* cysts found in a standard part of the brain of male and female mice infected with *Toxoplasma gondii*

Weeks following infection	Male	Female
1	—	—
2	51±1.8	53±1.6
3	120±3.67	127±3.0
4	213±2.8	215±2·9
5	358±2.9	366±3.02
6	435±3.9	445±4.2
Controls	—	—

The results of the semiquantitative assessment of the lesions in the various groups are shown in Table 2. There is a gradual, time-related increase in the severity of the lesions. The only exception is the group of female mice 6 weeks after inoculation, which showed slightly less severe changes than those seen in the previous group (5 weeks).

Slightly more severe changes are observed in females than in males of the same group (Table 2). However, the number of cysts in these groups shows no significant difference ($P>0.05$) (Table 1). These sex differences reflect the heavier lymphoplasmacytic infiltration in females. The number of cysts, microglial reaction, necrosis and distribution of the lesions do not appear to differ in the two sexes.

These results indicate that control mice and those killed 1 week following toxoplasmic infection showed no histological changes in the brain. After 2 weeks there is minimal perivascular or diffuse infiltration, mainly lymphoplasmacytic, with a few polymorphs, in all coronal sections of the brain, with no specific topographic predilection (Fig. 4). The meninges showed no significant inflammatory infiltration, while the rare toxoplasmic cysts were revealed only with the PAP method (Fig. 1).

Three weeks after inoculation all contained *Toxoplasma* cysts, some surrounded by a moderate cellular micoglial and inflammatory reaction with necrosis. In some areas the reaction was more intense and was accompanied by inflammatory infiltration of the overlying meninges. A lymphoplasmacytic infiltration was often observed in relation to the ventricles. Although the changes were seen in many areas of the brain, the medulla oblongata was most severely involved, while the cerebellum was almost unaffected. After 4 weeks the changes were more severe (see Table 2), especially in the medulla oblongata. However, in these groups there were also diffuse areas of intense inflammatory and necrotic changes,

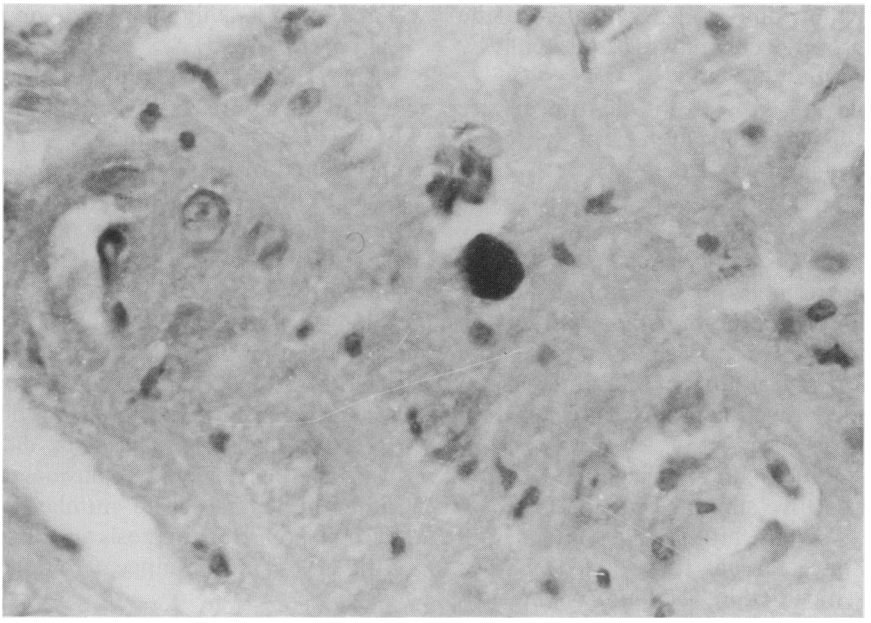

Fig. 1. Male mouse 2 weeks after inoculation with *Toxoplasma gondii*. A *Toxoplasma* cyst with no surrounding inflammatory infiltrate giving a strong positive reaction with the PAP method. × 125.

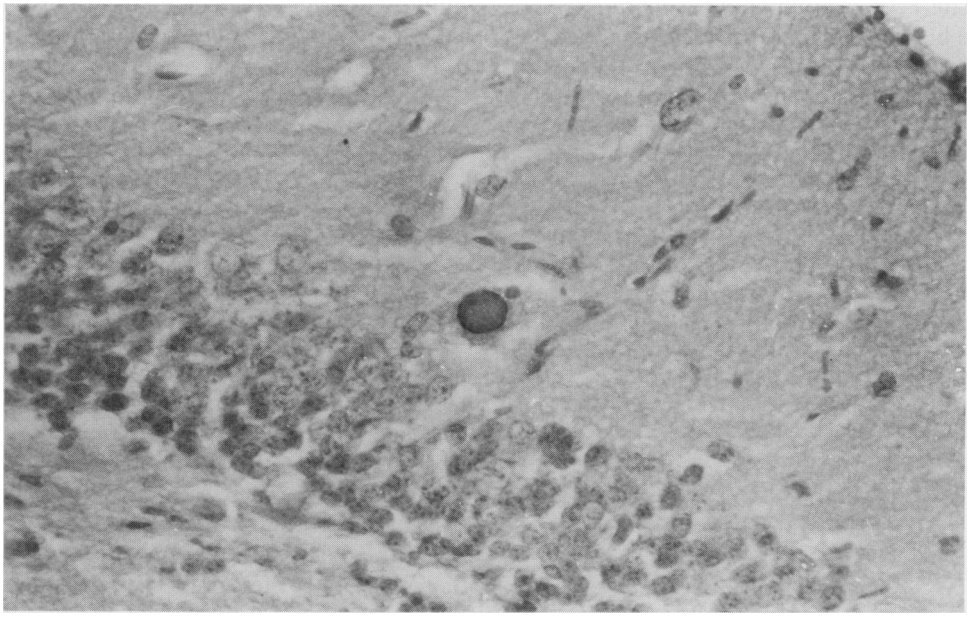

Fig. 2. Female mouse 2 weeks after inoculation. A *Toxoplasma* cyst close to nerve cells of a brain nucleus giving a node-rate positive reaction with the PAP method. × 125.

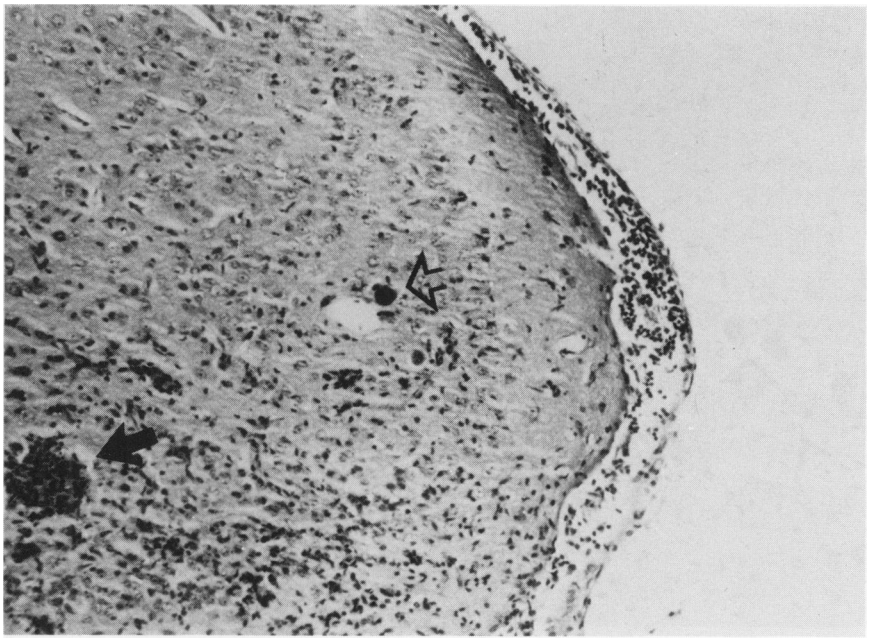

Fig. 3. Intense diffuse or nodular (solid arrow) microglial and inflammatory reaction of the brain. Lymphoplasmacytic infiltration of the meninges. The PAP method revealed occasional strongly positive *Toxoplasma* cysts (open arrow). × 80.

Table 2. Total score per group obtained by histological assessment of the lesions in the brains of mice after infection with *Toxoplasma gondii*

Weeks following infection	Histological score (maximum 25)	
	Male	Female
1	—	—
2	6	10
3	12	16
4	16	21
5	19	23
6	20	21
Controls	—	—

mostly in the frontal sections of the brain. The meninges contained numerous lymphocytes and plasma cells.

Five weeks following inoculation the brains showed meningeal lymphoplasmacytic infiltration and intense microglial and inflammatory reaction with cellular necrosis, either diffuse or forming reactive, space-occupying nodules (Fig. 3). Occasional dystrophic nodules with calcium deposits were also seen and heavy lymphoplasmacytic infiltration was present around the fourth ventricle (Fig. 5). Nevertheless, diffuse areas of necrosis and cellular reaction as well as numerous *Toxoplasma* cysts were evident with haematoxylin and eosin stains in various parts of the brain, including the cerebellum.

The changes in mice 6 weeks following toxoplasmic infection were similar to those described in the preceding group.

Discussion

The findings are substantially in agreement with those described by other authors (Stahl *et al.* 1966; Huldt 1966; Lindberg & Frenkel 1977).

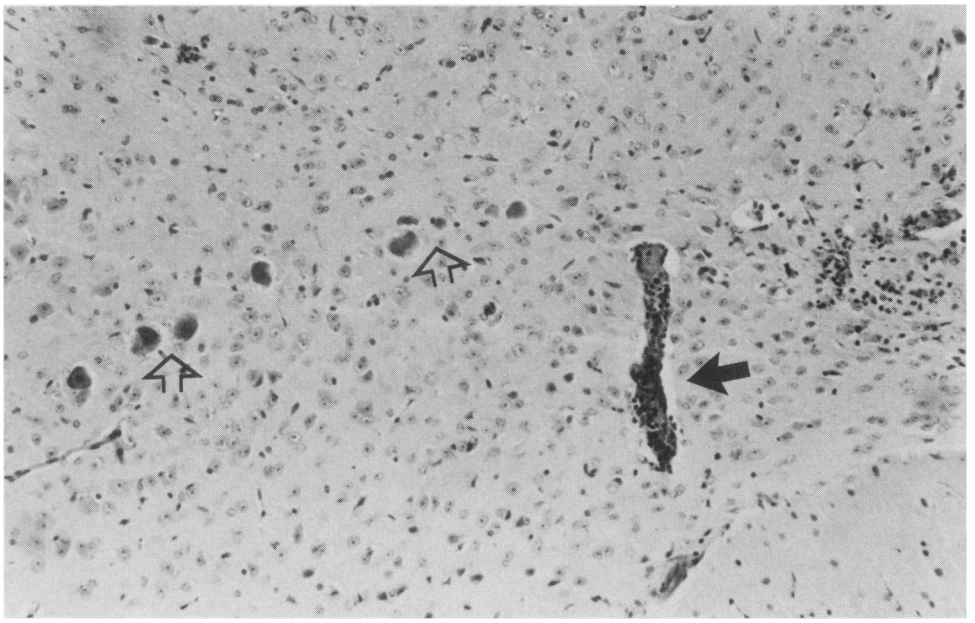

Fig. 4. Female mouse 2 weeks after inoculation. A mild, mainly perivascular inflammarory infiltrate (solid arrow). Open arrows show nerve cells which may be misinterpreted as *Toxoplasma* cysts with conventional stains. H & E × 80.

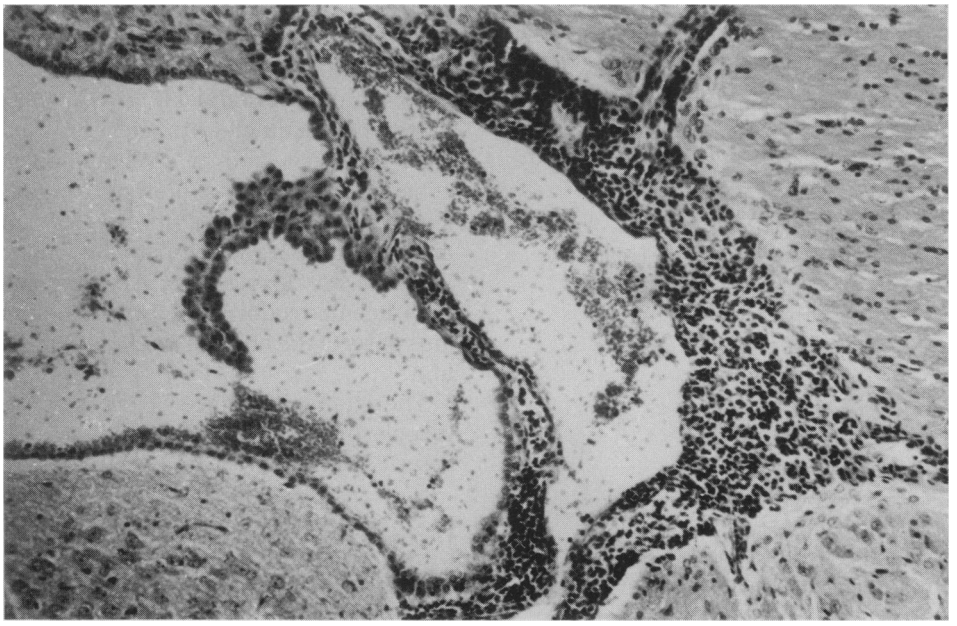

Fig. 5. Heavy lymphoplasmacytic infiltrate around the fourth ventricle of a female mouse 5 weeks after toxoplasmic inoculation. H & E × 80.

The perivascular inflammatory infiltration seen as early as 2 weeks after infection has been reported by Budzilovich (1961). Additionally, we have been able to demonstrate with the PAP method the early formation of *Toxoplasma* cysts on histological sections. This has not been previously reported. However, the number of cysts so demonstrated was small and unrelated to the inflammatory lesions. This finding is in accordance with the hypothesis of a major role of cellular immunity in the pathogenesis of toxoplasmic lesions even in non-lymphoid organs (Kittas & Henry 1979). This immunologically-based pathogenetic mechanism may also explain the previously reported observations of more severe lesions in female than in male experimental animals (Huldt 1966; Beverley & Henry 1971). These observations are confirmed by the present findings. However, in our experiment these sex differences reflect heavier lymphoplasmacytic reaction in the lesions of female mice and not genuine differences in microglial reaction, necrosis or number of cysts. It is possible that these findings may be explained by the better response of adult females in both cell-mediated and humoral immunity (Kenny & Gray 1971; Kittas & Henry 1980).

From the third week onward the lesions became gradually heavier and more widely distributed. The lesions were more intense during the fifth week, when even dystrophic calcium deposits were also observed. In man, such deposits are considered as a sign of long term illness (Cheever *et al.* 1965).

The topographic study of the lesions showed the medulla oblongata and the area surrounding the fourth ventricle to be the most severely affected. This correlates with the occurrence of obstructive hydrocephaly, which is not only seen in congenital (Altshuler 1973), but also in acquired toxoplasmosis (Best & Finlayson 1979).

The cerebellum was rarely involved and less severely damaged, a finding which is in contrast to the manifestations of human toxoplasmic CNS involvement (Greenlee *et al.* 1975). However, the diffuse cerebral and meningeal involvement correlates well with the clinical manifestation of human toxoplasmic meningoencephalitis (Hafström 1959), while the formation of local reactive nodules, which may reach a significant diameter, would explain the clinical symptomatology of toxoplasmic space-occupying lesions (Hussey 1975).

The advantages of the immunohistochemical techniques in the study of toxoplasmosis are shown, both in the non-nervous organs and in the CNS. The fact that toxoplasmic elements can be demonstrated with the PAP method in paraffin sections of routinely processed material may prove to be a great advantage in the study of surgical material as well as in retrospective studies, such as the evaluation of the probable toxoplasmic cause of intrauterine deaths.

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