IMMUNOHISTOCHEMICAL STUDY OF ACUTE AND CHRONIC TOXOPLASMOSIS IN EXPERIMENTALLY INFECTED MICE

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Abstract. Acute and chronic *Toxoplasma* infections were evaluated in mice using stage specific antibodies and immunocytochemistry. Mice with acute toxoplasmosis were less active, had erectile body hair and seldom took food or water resulting in weight loss. All mice died within 7 days post-inoculation. The immunohistochemical technique enhanced visualization of parasites allowing their distribution to be accurately followed. Following intraperitoneal infection, tachyzoites were initially identified on the surface of the liver and spleen. There was a rapid increase in the number of tachyzoites associated with invasion from the surrounding connective tissue into the organs with formation of inflammatory lesions in the liver. The focal inflammatory lesions showed increasing numbers of tachyzoites with the period post-inoculation. Similar increases in tachyzoites were observed for the spleen. In contrast, only a few individual tachyzoites were seen in the brain at the final time point. In chronic infections, the mice were asymptomatic but tissue cysts containing large numbers of bradyzoites were observed in all brains with the average number of 295 tissue cysts per half brain and the average cystic size of 46.02 ± 5.08 μm. By histology and immunostaining, the tissue cysts were readily identifiable along with a mild inflammatory cell infiltration into the meninges and perivascular cuffing. Double immunocytochemical labelling confirmed the exclusive presence of tachyzoites during the acute phase and bradyzoites during the chronic phase.

INTRODUCTION

Toxoplasma gondii is an intracellular parasite that infects a wide variety of hosts, including humans. Infection generally occurs through ingestion of either oocysts shed in cat feces or viable tissue-cysts in undercooked meat. In addition, primary infection during pregnancy can result from transplacental transmission which can result in severe congenital disease in the fetus with potential abortion (Coppin *et al*, 2003), severe birth defects, including hydrocephaly, calcification, neurological defects and chorioretinitis which may be recurrent (Wong and Remington, 1994). During acute infection, tachyzoites are the rapidly multiplying stage of the parasite. They can invade and proliferate in all nucleated cells by active penetration and form parasitophorous vacuoles. After repeated replication, host cells are disrupted and tachyzoites disseminate via the bloodstream and can invade many tissues, including the central nervous system, eye, skeletal and heart muscles and placenta. Replication leads to cell

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death and rapid invasion of neighboring cells. The tachyzoite stage causes a strong inflammatory response and tissue destruction and, therefore, causes clinical manifestations of disease (Montoya and Liesenfeld, 2004). The RH strain is a type 1 virulent strain causing severe toxoplasmosis in the mouse model suggesting that it may also be more pathogenic in humans (Mordue *et al,* 2001). However, with the onset of the host immune response, a subpopulation of tachyzoites in the brain undergoes stage conversion to bradyzoites which multiply slowly to form large tissue cysts with many thousands of bradyzoites capable of persisting for the life of the host (Mordue *et al,* 2001; Montoya and Liesenfeld, 2004). In immunocompromized hosts, it is believed that reactivation, probably due to cyst rupture, results in reversion from dormant bradyzoites into rapidly dividing tachyzoites resulting in serious neurological disease. In the present study, acute and chronic toxoplasmic clinical manifestations as well as pathological features were compared. Apart from conventional histophatology, we used the immunohistochemical technique combined with stage (tachyzoite and bradyzoite) specific antibodies to enhance visualization of parasites during acute and chronic toxoplasmosis in mice.

MATERIALS AND METHODS

Animals

Nineteen ICR mice of both sexes, aged 5-6 weeks and with an average weight of 30-40 g were used in both acute and chronic studies. They were supplied by the National Laboratory Animal Center, Mahidol University, Thailand. The documentary proof of Ethical Clearance by the Faculty of Tropical Medicine-Animal Care and Use Committee (FTM-ACUC 004/2005), Mahidol University was approved on 17/02/2005.

Toxoplasma gondii strains

The virulent RH strain which was maintained in our laboratory by continuous passage every 3-4 days in mice for 15 years was used to produce acute infection. The avirulent O'Toole strain, provided by the Toxoplasma Unit, Public Health Reference Laboratory, Swansea, United Kingdom, was used in the chronic study.

Antibodies

The stage antibodies, rabbit anti-SAG1 specific for tachyzoites (Harning *et al,* 1996) and mouse anti-BAG1 specific for bradyzoites (Bohne *et al,* 1995) as well as the rat antibody CC2 which recognizes the tissue cyst wall (Gross *et al,* 1995) were used.

Acute infection study

Twelve mice were infected by the intraperitoneal inoculation of 5,000 RH tachyzoites of *T. gondii*. Groups of four mice were autopsied at 3, 5 and 7 days post-inoculation.

Chronic infection study

Seven mice were infected by oral feeding of ten tissue cysts. The mice were then autopsied at 48 days post-inoculation.

Autopsy

In the acute study, the liver, spleen and brain were removed and fixed in 2% paraformaldehyde in 0.1M phosphate buffer for histology, whilst in the chronic study the brain was removed and divided into two equal halves. One half was fixed in paraformaldehyde for histology, while the other was homogenized and used for counting the number and measuring the size of the tissue cysts.

Histology

The fixed tissue was dehydrated and embedded in wax. Sections were cut and stained with hematoxylin and eosin.

Immunocytochemistry

Sections of tissue from the acute and chronically infected mice were picked up with slides coated with 2%, 3-aminopropyl triethoxyl silane. Sections were dewaxed and endogenous peroxidase blocked prior to us-

ing pressure cooking for antigen retrieval (Ferguson, 2004).

For peroxidase staining, the sections were exposed to the tachyzoite specific rabbit-anti-SAG1 or the bradyzoite specific mouse anti-BAG1 in TRIS buffer. They were washed and then exposed to the secondary antibody, either goat anti-rabbit immunoglobulins (Ig) or goat anti-mouse Ig conjugated to peroxidase. Labelling was visualized using diaminobenzidine and hydrogen peroxide as chromogen, then counter stained with hematoxylin prior to examination.

For double labelled immunofluorescence, sections were pretreated as above but then exposed to a mixture of rabbit anti-SAG1 and mouse anti-BAG1. These antibodies were then visualized using goat anti- rabbit Ig conjugated to FITC and goat anti-mouse Ig conjugated to Texas red. In addition, certain sections were also stained with anti-BAG1 and the rat-CC2 antibody and visualized using goat anti-mouse Ig conjugated to FITC and goat anti-rat Ig conjugated to Texas red. The sections were examined with a Zeiss Axioskop microscope, and pictures were taken with a cooled CCD camera using Improvision Openlab software running on an Apple Macintosh computer.

Data analysis

The number and size of the liver lesions were measured. These results were then evaluated statistically using Mann-Whitney *U* test, and using Kruskal-Wallis test when comparing to the data among different groups.

RESULTS

Mice with acute toxoplasmosis showed obvious clinical manifestations such as being less active and their body hair standing on end. They seldom took food or water and lost weight. All mice died within 7 days post-inoculation (DAI). Histology showed the surface of the liver being affected with several small inflammatory lesions, the number of which increased and progressed into deeper areas through time post-inoculation (Fig 1a, b, Table 1). There was a significant increase in the number ($p=0.001$) but no significant increase in the size ($p=0.152$) of the lesions. However, while it is difficult to identify individual parasites in histological sections (Figs 1a, b, 2c), they were easily identified after immunohistochemical staining (Figs 1c, 2a, b, d-f). This allowed the identification of the small numbers of parasites associated with the connective tissue on the surface of the organs during the early stages of infection at 3 DAI (Figs 2a, b and inserts). Using this technique, it was possible to observe changes in the number and distribution of the parasites. It was found that there was a massive increase in the numbers of parasites associated with the connective tissue at 5 DAI (Fig 2d) compared to 3 DAI (Fig 2b). The parasites were also present along the connective

Table 1
The number and size of the liver lesions and the number of inflammatory cells in acute toxoplasmosis.

Day after infection (DAI)	Average number of the liver lesion	Size of the liver lesions (μm)	Average number of inflammatory cells			
			E	N	M	Total
3 DAI	2.3[a]	57.25 ± 24.82[b]	0.50	3.50	6.88	10.88
5 DAI	10.3[a]	59.58 ± 15.80[b]	0.04	1.61	5.39	7.04
7 DAI	58[a]	65.33 ± 17.29[b]	0.20	0.70	4.37	5.23

E = Eosinophil, N = Neutrophil, M = Mononuclear cell
[a]= p-value <0.05, [b]= p-value >0.05

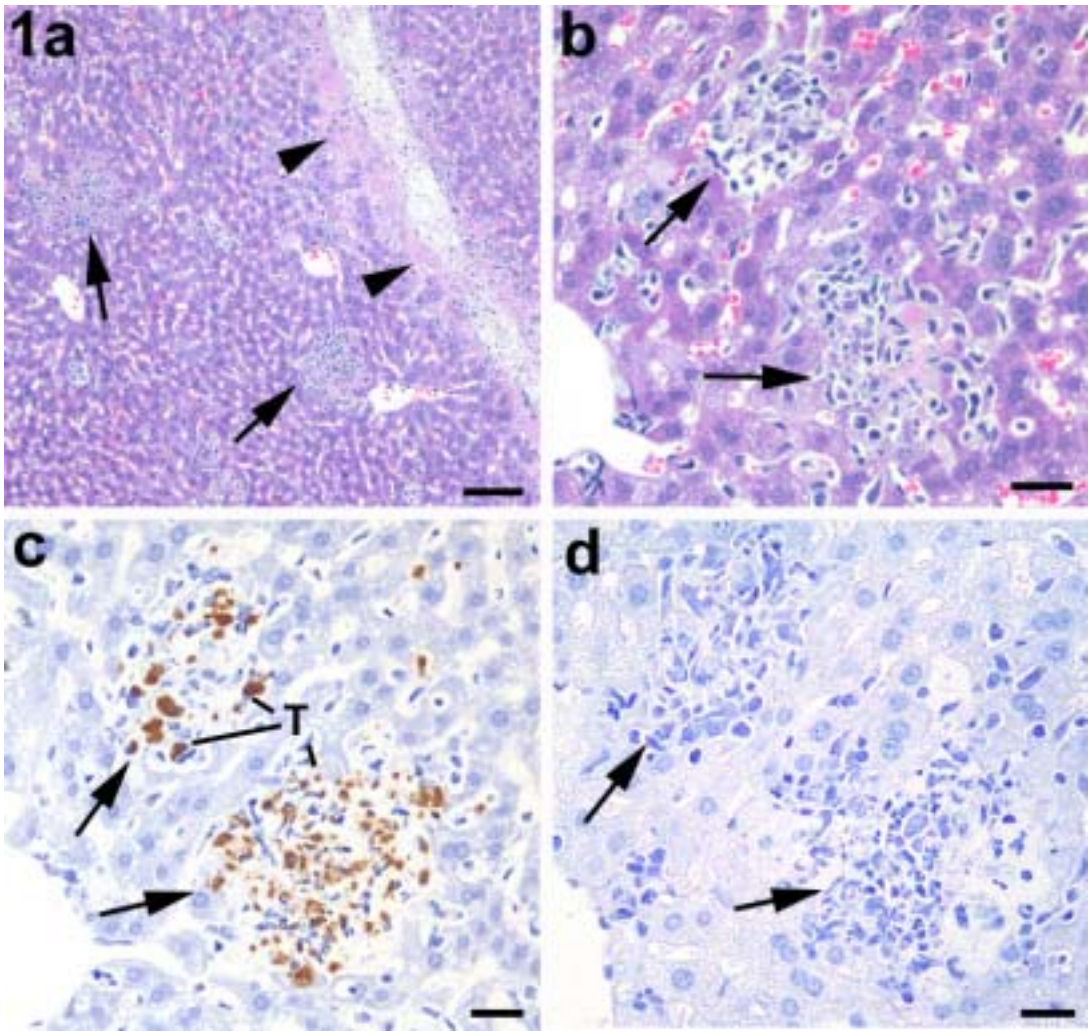

Fig 1–Sections from the liver of acutely infected mice: a) Low power image of a section through the liver at 5 DAI showing a number of small lesions (arrows) and a strand of connective tissue (arrowheads) with numerous parasites which cannot be identified in the H & E stained section. Bar is 50 μm; b-d) Serial sections of two small inflammatory lesions (arrows) stained with H & E (b) Immunostained with anti-SAG1 using peroxidase either with pressure coking (c) or without pressure cooking (d) In the H & E stained section, it is difficult to identify the individual parasites, whilst large numbers of positive brown staining parasites could be easily seen in the immunostained section pretreated with pressure cooking. In contrast, in the identically treated section without pressure cooking the parasites appeared unstained. Bars are 20 μm.

tissue strands within the liver. Initial inflammatory lesions had few parasites but the number rapidly increased with time with numerous present at 5 DAI (Fig 1c). In addition, a number of hepatocytes were also infected and a number of these contained large groups of parasites (Fig 2e). These bore some resemblances to tissue cysts but were confirmed as tachyzoites by being SAG1 positive and BAG1 negative (Fig 2e). Similar parasitic invasion and proliferation were observed in the spleen (Fig 2d). In contrast, no parasites were observed

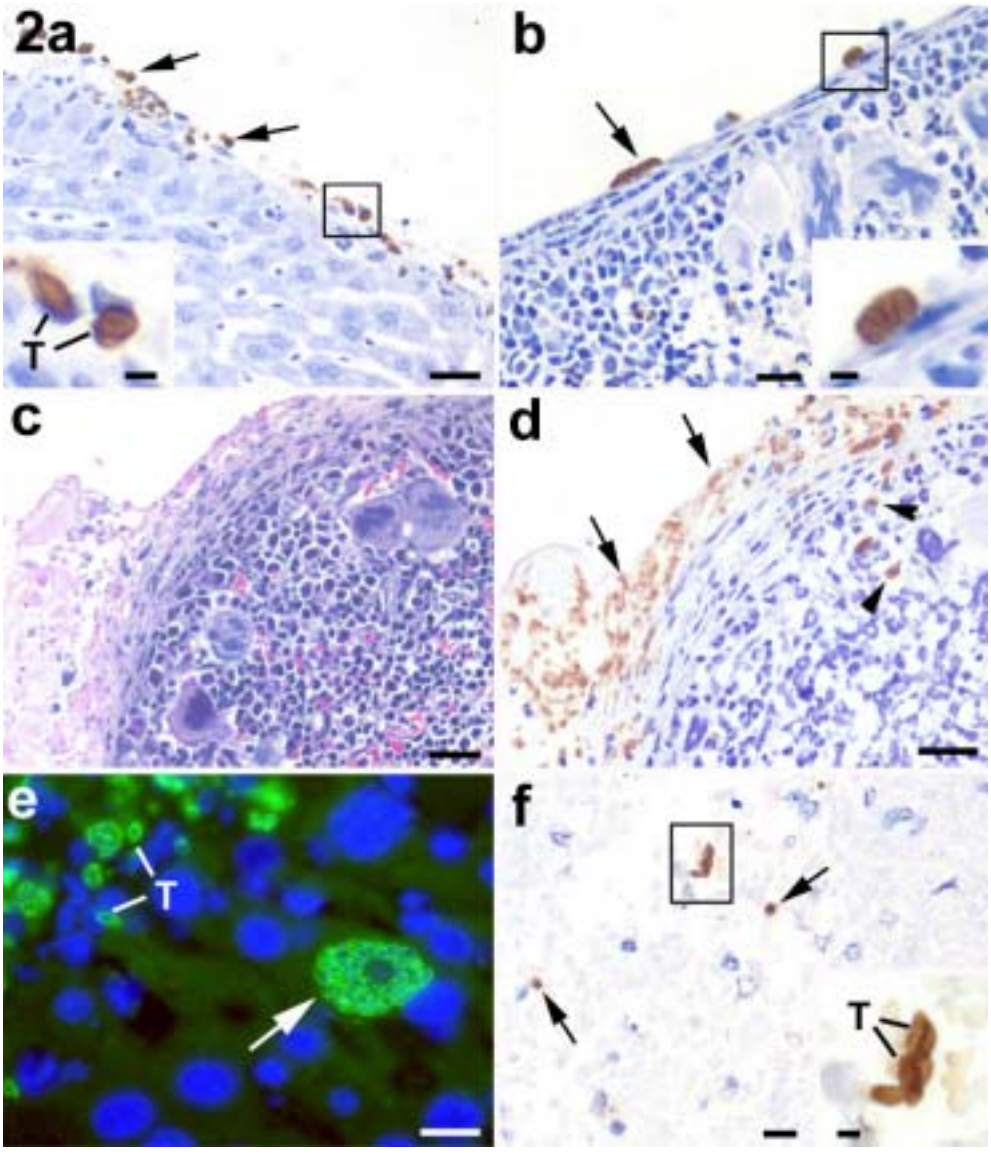

Fig 2–Sections from the liver and spleen of acutely infected mice: a-b) Sections at an early stage of infection (3 DAI), immunostained with anti-SAG1 showing a number of positively stained parasites (arrows) associated with the connective tissue enclosing the liver (a) and spleen (b). Bars are 20 μm. Inserts) Enlargement of the enclosed areas showing the positively brown stained tachyzoites (T). Bars are 1 μm; c-d) Serial sections through the spleen at a later stage of infection (5 DAI) either H & E stained (c) or immunostained. (d) Note the large numbers of parasites within the connective tissue (arrows) and also within the spleen (arrowheads) that can be identified in the immunostained section. Bars are 20 μm; e) Immuno-fluorescent stained section double labelled with anti-SAG1 (green) and anti-BAG1 (red) through the periphery of a liver lesion at a similar stage of infection to that in Fig 1. Note the numerous tachyzoites (T) associated with the lesion and that the large clump of parasites (arrow) is also exclusively tachyzoites. Bar is 10 μm; f) Section through the brain at 7 DAI immunostained with anti-SAG1 showing a few tachyzoites located in the brain parenchyma (arrows). Bar is 20 μm. Insert) Detail of the enclosed area showing the positive brown stained tachyzoites (T). Bar is 1 μm.

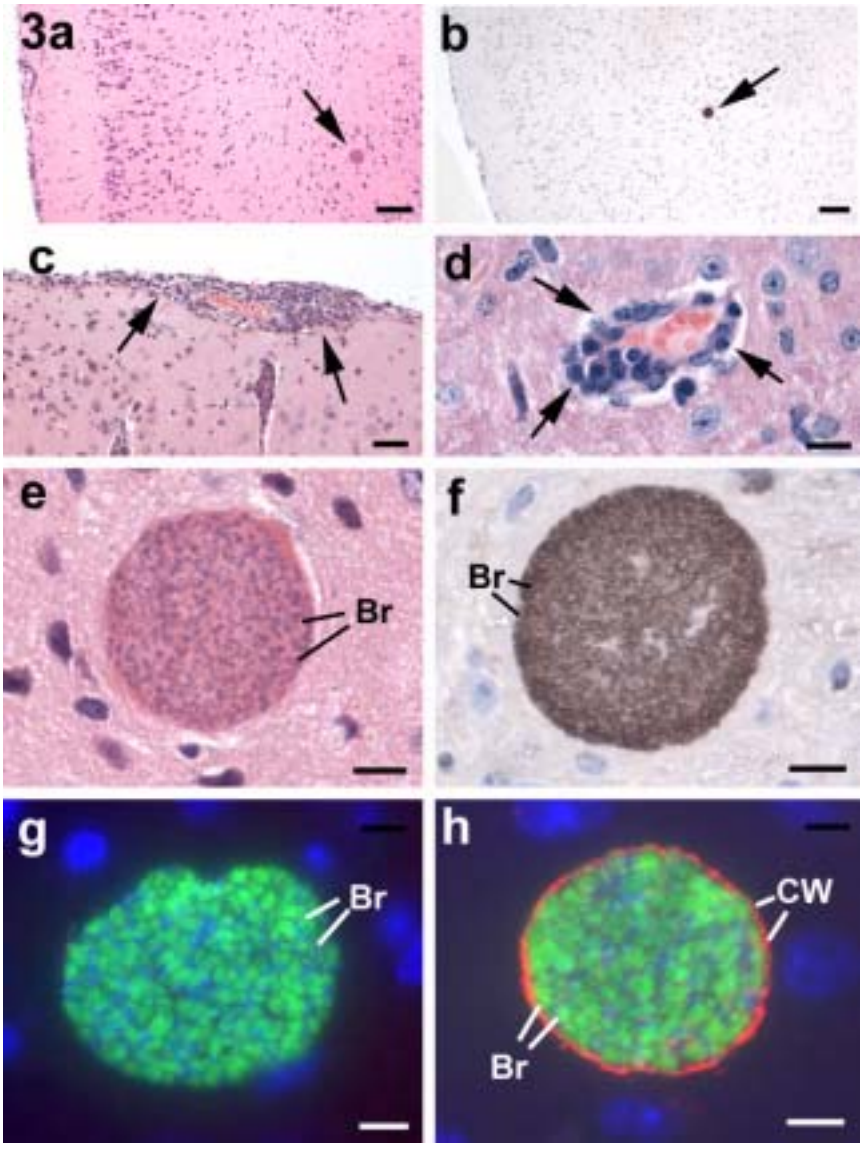

Fig 3–Sections from the brains of chronically infected mice: a-b) Low power images showing that it is possible to identify tissue cysts in both H & E (a) and immunostained (b) sections (arrows). In the section immunostained with anti-BAG1 the strongly brown staining tissue cyst stands out against the pale blue background (arrow). Bars are 100 μm. c) Part of the periphery of the brain shows a number of inflammatory cells located within the meninges (arrows). H & E stained. Bar is 50 μm. d) Detail showing a blood vessel surrounded (cuffed) by inflammatory cells (arrows). H & E stained. Bar is 10 μm. e) Detail of a tissue cyst containing large numbers of bradyzoites (Br). H & E stained. Bar is 10 μm. f) Tissue cyst immunostained with BAG1 and peroxidase showing the strong cytoplasmic staining of the bradyzoites (Br). Bar is 10 μm. g) Immuno-fluorescent stained section double labelled with anti-BAG1 (green) and anti-SAG1 (red) showing the BAG1 positive bradyzoites (Br) within the tissue cyst and the absence of any SAG1 positive tachyzoites. Bar is 10 μm. h) Immuno-fluorescent stained section double labelled with anti-BAG1 (green) and antibody CC2 (red) showing the BAG1 positive (green) bradyzoites (Br) within the tissue cyst enclosed by CC2 positive (red) cyst wall (CW). Bar is 10 μm.

in the brain, except for a few SAG1 positive tachyzoites seen on day 7 (Fig 2f, insert).

In the chronic infection study, mice were asymptomatic. Quantitation of half of the brain showed an average of 295 tissue cysts with an average size of 46.02 ± 5.08 μm. Histological examination of the brain identified a few tissue cysts containing large numbers of parasites (Fig 3a, e) which were also readily identifiable in immunostained sections (Fig 3b). In addition, some inflammatory cells were observed within the meninges (Fig 3c) with perivascular cuffing (Fig 3d), but inflammatory cells were not associated with intact tissue cysts. Immunohistochemistry confirmed that the tissue cysts contained BAG1 positive bradyzoites (Fig 3f). In double labelled sections, while BAG1 positive cysts could be identified, SAG1 positive tachyzoites were absent (Fig 3g). The presence of the tissue cyst wall surrounding the bradyzoites could be confirmed by double labelling with the anti-BAG1 and CC2 (Fig 3h).

In the present study, the sections were pre-treated by pressure cooking prior to immunostaining. The antigen retrieval technique significantly improved the quality of the immunostaining as shown in the identically stained serial sections of the liver where the numerous parasites are stained brown in the pressure cooked section (Fig 1c) but are unstained in the conventional treated section (Fig 1d).

DISCUSSION

There are several techniques used to diagnose toxoplasmosis including serology and tissue pathology. Routine histological stains such as hematoxylin-eosin (Conley *et al,* 1981; Wanke *et al*, 1987), Giemsa (Conley *et al*, 1981; Wanke *et al*, 1987), eosin-methylene blue (Derouin *et al*, 1989) or periodic acid-Schiff (Conley *et al*, 1981) have been used to visualize parasites. However, the tachyzoites or cysts are often difficult to identify especially when present in low numbers. Even in lesions, it is difficult to appreciate the number of parasites present. The usefulness of immunohistochemistry using stage-specific antigens and peroxidase staining for identifying parasites described in the present study is similar to that described previously (Byron, 1997; Shi *et al*, 2001; Ferguson, 2004). The development of antigen retrieval techniques such as pressure-cooking technique enhances the specific staining of the parasites. The technique is based on the high temperature associated with pressure cooking resulting in the unmasking of the antigenic sites masked by fixation and wax embedding thus improving antibody recognition (Shi *et al*, 2001; Ferguson, 2004).

In recent years, much emphasis has been placed on differentiating between these stages and understanding stage conversion. This has been assisted by the molecular characterization of a number of molecules expressed exclusively in either the tachyzoite or bradyzoite (Lyons *et al*, 2002; Gross *et al*, 2004; Ferguson, 2004). In the present study, an antibody to SAG1 was used to identify tachyzoites and BAG1 to identify bradyzoites, since there is rapid on-off switching of these molecules during stage conversion (Ferguson, 2004). In a previous study of acute toxoplasmosis, we demonstrated severe inflammation and necrosis of the liver, spleen and pancreas but, tachyzoites could not be demonstrated in the brain, although mild to moderate congestion was observed in the brain parenchyma and meninges (Sukthana *et al*, 2003). In the present study, using immunohistochemistry and SAG1 antibody, it was possible to identify tachyzoites not only in the liver and spleen but also in the brain during acute toxoplasmosis. Similarly, the technique is useful in identifying the low number of tissue cysts present in brain sections in chronically infected mice.

In this study, intraperitoneal infection by the virulent RH stain of *T. gondii* was used. It is an abnormal route of infection since, in na-

ture, animals are normally infected by ingesting sporozoites within oocysts or bradyzoites within tissue cysts with almost unnoticeable clinical manifestation. Signs and Symptoms of acute toxoplasmosis could be obtained either by orally infected mouse with tissue cysts from high-virulent RH strain or by oral application of low-virulent *T. gondii* cysts in immunosuppressive mice. Deckert-Schluter and colleagues (1998a,b) orally infected mice lacking the IFN-gamma-receptor and the TNF-receptor type 1 (TNFR1) with low-virulent *T. gondii* cysts and found that such mice were highly susceptible to *T. gondii* and died of acute fulminant toxoplasmosis. Among the various organs affected, hepatitis was severe enough to cause death.

Based on reports that the RH strain, after passage for long time or after more than 35 intraperitoneal passages of tachyzoites in mice, had lost both the capacity to form oocysts and a marked reduction or absence of tissue cysts formation (Frenkel *et al*, 1976; Dubey *et al*, 1999), we employed the RH strain tachyzoites in our laboratory for more than 35 passages (longer than 15 years). Thus we believed our RH strain had become a cystless and oocystless strain. Moreover, since the tachyzoite stage was immediately inactivated by pepsin and acid in the stomach (Jacobs *et al,* 1960; Dubey, 1998), we decided to produce acute toxoplasmosis by intraperitoneal (IP) infection with tachyzoite stage. We succeeded as evidenced by acute clinical manifestations in all infected mice and their correlation with pathological features. However, because of IP route, the tachyzoites were observed to rapidly proliferate and directly extend from the outer surface of the liver and spleen causing several small inflammatory lesions and then spreading into deeper area with more severe pathological features noted later on. It is noteworthy that these findings are contrary to the natural route of infection which is the result of hematogenous spread through the portal system in the case of the liver, and that lesions are due to expansion from the inner to the outer surface of internal abdominal organs (Dubey *et al*, 1997). Thus there can be marked differences in the progress of parasitic dissemination between the different methods of infection.

The bradyzoites in tissue cysts are considered benign. It was noted that the tissue cyst did not illicit an immune response although there were a number of inflammatory cells within the meninges and cuffing the blood vessels. While it is possible to identify tissue cysts in conventionally stained sections, this can be time consuming. In contrast, the tissue cysts can be easily recognized at low magnification in immunostained sections. Moreover, double labeling immunocytochemistry is invaluable in studying stage conversion between bradyzoites and tachyzoites (Ferguson, 2004). They can also be useful in the diagnosis of *T. gondii* infection in biopsies of immunocompromized patients (Ferguson, unpublished observations).

ACKNOWLEDGEMENTS

This work was financially supported by the Thailand Research Fund through Royal Golden Jubilee Scholarship. We are grateful to the Immunohistochemistry Unit, John Radcliffe Hospital, Oxford, UK for providing the facilities and equipments used in this study. We are indebted to Dr E Guy for supplying the avirulent O'Toole strain and to Drs U Gross and E Petersen for providing the antibodies. The authors would like to thank Mr Saiyood Incheng for taking care of the experimental mice.

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