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# Antigen specific lymphocyte transformation induced by secreted antigens from *Toxoplasma gondii*

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## SUMMARY

Secreted (TSA) and water lysed (WLA) antigens derived from cell culture of the RH strain of *Toxoplasma gondii* have been used to induce antigen specific mitogenesis of lymphocytes from patients with symptomatic and asymptomatic toxoplasmosis. Lymphocyte responsiveness to WLA was similar to previous reports, with about 50% of patients showing a false negative reaction. Responses to TSA however were highly specific, with no false negative reactions. This increased specificity was not due to an increased response against TSA by patients' lymphocytes ($P < 0{\cdot}001$), but a lower TSA response by uninfected subjects' lymphocytes ($P > 0{\cdot}1$) compared with WLA in both cases. In a minority of both infected and uninfected subjects, there was a low but detectable response to antigens secreted by the host cell line (HCA), and this was directly compared to their responses against TSA. There was at least a 10-fold increase in the patients' responses to TSA when compared with HCA ($P < 0{\cdot}001$), whereas there was no significant difference between the uninfected subjects' responses to these antigens ($P > 0{\cdot}1$). Preliminary observations have suggested that TSA is distinct from other defined secreted antigens as both heat treatment and solid phase immunosorption did not have any noticeable effect on TSA-induced mitogenesis.

**Keywords** *Toxoplasma gondii* cell-mediated immunity diagnosis secreted antigens lymphocyte transformation

## INTRODUCTION

In both congenital and disseminated toxoplasmosis, serologial indicators of infection are not always reliable. In countries where antenatal serological screening is not obligatory, as is the case in the UK, there is a need for rapid and accurate diagnosis in the newborn so that appropriate decisions may be taken for clinical management and prognosis. Serological diagnosis has been shown to be unreliable due to the transplacental passage of maternal IgG and the high incidence of false negative IgM reactions in the newborn (Wilson *et al.*, 1980). In disseminated disease, serology has severe limitations due to the presence of high, stable antibody titres during an opportunistic infection which often bear little or no relationship to the severity of disease (Gransden & Brown, 1983; Remington, 1982).

Resistance to a *Toxoplasma* infection is thought to be primarily mediated by the cellular immune response (Krahenbuhl & Remington, 1982), in which the activation of macrophages for intracellular killing is thought to play a central role in host defence (Remington, Krahenbuhl & Mendenhall, 1972). Tests for cell-mediated immunity such as delayed type hypersensitivity (DTH)

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*in vivo* and the lymphocyte transformation test (LTT) *in vitro* have been used for the detection of acquired and congenital toxoplasmosis (Frenkel, 1948; Krahenbuhl, Gaines & Remington, 1979). Although the LTT shows a general agreement with infection in the newborn and in chronically infected adults, as detected using serological and DTH criteria, lymphocytes from acutely infected patients (i.e. with infections of $<9$ months' duration) do not always proliferate in response to a conventional *Toxoplasma* lysate antigen (Anderson, Krahenbuhl & Remington, 1979).

Secretions from *Toxoplasma* appear to have major roles in host cell invasion, parasite survival and cyst formation (Matsubayashi & Akao, 1966; Nichols, Chiappino & O'Connor, 1983). Secreted antigens are also present in the circulation during acute infection (Hughes, 1981; Hughes & Van Knapen, 1982) and are indicators of an active infection. Accordingly, experiments have been carried out to ascertain whether secreted antigens from *Toxoplasma* are able to induce antigen specific lymphocyte transformation.

These preliminary investigations show that *Toxoplasma* antigens present in *in vitro* culture supernatants are able to induce a specific mitogenic response in a random sample of chronically infected patients, and suggest that the antigen(s) responsible is different from other previously defined secreted antigens.

## MATERIALS AND METHODS

*Host and parasite cell culture.* Primary cultures of African green monkey kidney cells (AGMPK) were purchased from Flow Laboratories (Irvine, UK) and grown in RPMI 1640 medium containing 2 mM glutamine, 100 u/ml penicillin, 100 $\mu$g/ml streptomycin and 10% fetal bovine serum (RPMI). Cells were subcultured at a ratio of 1:4 every 4–5 days and usually survived for up to 1 month of continuous culture, at which time new cultures were initiated from liquid nitrogen stocks.

*Toxoplasma gondii* (RH strain) was originally obtained from outbred AVR mouse (Animal Virus Research Institute, UK) peritoneal fluid 3 days after i.p. infection. The *Toxoplasma* suspension was washed three times in Hanks' balanced salt solution (HBSS) by centrifugation (150*g* for 10 min at 4°C) and seeded onto a confluent culture (75 $cm^2$ culture area) of AGMPK cells in 20 ml RPMI at a final density of $5 \times 10^5$ parasites/ml. After 96 h of culture at 37°C, the flasks were gently agitated and the supernatant removed. Typically, 20 ml of supernatant contained up to $10^9$ viable *T. gondii* organisms, with $<2\%$ host cell contamination. Control supernatants were collected from uninfected AGMPK cells grown under identical conditions.

*Antigen preparation.* Supernatants from *T. gondii* infected or control uninfected AGMPK cells were clarified by centrifugation and used to prepare *Toxoplasma* secretory antigens (TSA) or host cell control (HCA). Conventional, water lysed antigens (WLA) were prepared from *T. gondii* recovered from the former supernatants, as described by Krahenbuhl & Remington (1979). The full protocol for each antigen preparation is shown in Fig. 1. The protein concentrations of each preparation was determined by the method of Lowry *et al.* (1951), prior to storage at $-70$°C.

*Lymphocyte transformation.* Assays were performed using techniques described in principle elsewhere (Wilson *et al.*, 1980). Briefly, peripheral blood was taken by venupuncture into preservative-free heparin and mononuclear cells separated from plasma by density gradient centrifugation (Lymphocyte Separation Medium, Flow). Cells were washed twice in HBSS by centrifugation (150*g* for 10 min) and resuspended in complete RPMI culture medium ($1{\cdot}14 \times 10^6$ cells/ml). Triplicate cultures of $2 \times 10^5$ cells/ well were prepared in U form microwell culture plates by the addition of 175 $\mu$l of cell suspension and 25 $\mu$l of antigen or mitogen to produce the following final concentrations: WLA and PHA (25, 12·5, 6·25 $\mu$g/ml); HCA and TSA (100, 50, 25, 12·5 $\mu$g/ml); Con A (50, 25, 12·5 $\mu$g/ml). After 120 h (with antigen) or 72 h (with mitogen) of culture at 37°C in a humidified atmosphere containing 5% $CO_2$ in air, the mitogenic response was assessed by the addition of 0·5 $\mu$Ci $^3$H-[methyl]-thymidine ($^3$H-TdR) (specific activity 2·5 Ci/mmol) in 25 $\mu$l phosphate-buffered saline (PBS). After a further 4 h at 37°C, cells were harvested onto glass fibre filters using an automated cell harvester and prepared for $\beta$-spectrometry.

*Serodiagnosis.* Plasma from all infected and control individuals was tested for the presence of anti-*Toxoplasma* antibodies by the *Toxoplasma* Reference Laboratory of the Public Health

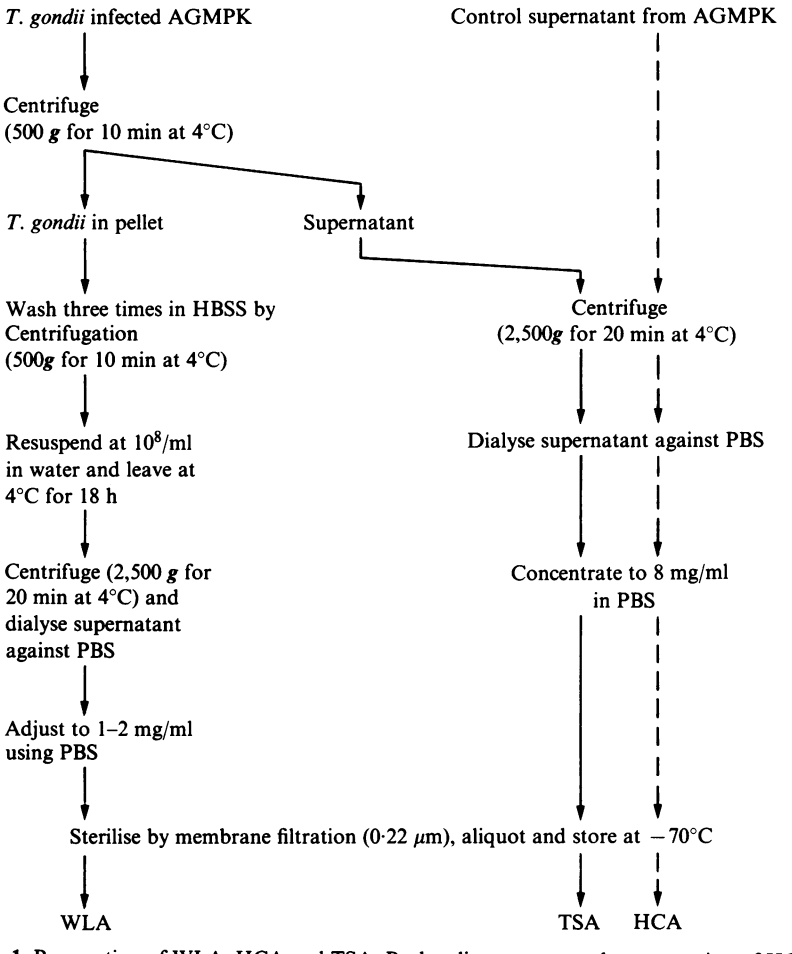


**Fig. 1.** Preparation of WLA, HCA and TSA. Broken lines represent the preparation of HCA.

Laboratory Service at St George's Hospital using the following assays: Sabin–Feldman dye test and Latex agglutination test as described by Balfour *et al.* (1982), IgM immunofluorescent test (IgM-IFA) carried out on plasma that had previously been fractionated to obtain IgM using a small gel filtration column (Ordones, Newman & Stone, 1982). All samples showing a positive reaction in the IgM-IFA were also screened for anti-immunoglobulin antibodies and found to be negative.

*Subjects.* Each of the 22 patients used in this study had significant titres of anti-*Toxoplasma* antibodies by one or more of the above tests. Clinical and serological data of patients and controls are summarized in Table 1.

## RESULTS

The established cell lines MRC5, VERO and HEp2 were tested for their ability to promote *Toxoplasma* growth *in vitro*. While all three cell lines were adequate in supporting *Toxoplasma* growth, the yields and purity of parasites was poor when compared to AGMPK cells (data not shown).

Following 4 day culture with AGMPK cells, infected cultures yielded a purity of *Toxoplasma* in excess of 98% with contamination by host cells of $<2\%$, and a 150-fold increase in parasite

**Table 1.** In all patients, except OF14 and OO14, symptoms were no longer apparent at the time of entry into this study

| Patient | Age | Sex | Symptoms | Serological reaction | | |
|---|---|---|---|---|---|---|
| | | | | Titre | | |
| | | | | Latex | Dye (iu/ml)* | IgM-IFA |
| FO07 | 39 | F | Fever, lymphadenopathy | ND | 1/512 (125) | + |
| MC11 | 39 | F | Fever, lymphadenopathy | 1/256 | 1/512 (125) | + |
| FO12 | 30 | F | Lymphadenopathy | 1/128 | 1/128 (31) | + |
| OF14 | 27 | F | Listlessness, Lymphadenopathy | > 1/8,192 | > 16,384 (8,000) | + |
| G107 | ND | F | Lymphadenopathy | 1/2,048 | 1/512 (2,000) | + |
| GR07 | 32 | F | Lymphadenopathy | 1/4,096 | 1/1,024 (250) | – |
| GR16 | 29 | F | Lymphadenopathy | 1/256 | 1/8,192 (4,000) | – |
| GO16 | 32 | F | Lymphadenopathy | 1/512 | 1/2,048 (1,000) | – |
| LY23 | 33 | F | Lymphadenopathy | 1/4,000 | 1/4,096 (2,000) | – |
| MR23 | 26 | F | Lymphadenopathy | 1/256 | 1/1,024 (500) | – |
| DO12 | ND | M | Lymphadenopathy | ND | 1/256 (62) | ND |
| ED12 | ND | M | Lymphadenopathy | ND | 1/1,024 (250) | ND |
| JO08 | 45 | F | Recurrent lymphadenopathy | 1/512 | 1/256 (62) | – |
| DA23 | 48 | M | Listlessness biopsy +ve | 1/512 | 1/1,024 (2,000) | – |
| CA16 | 47 | F | Listlessness | 1/1,024 | 1/8,192 (4,000) | – |
| MA16 | 41 | F | Uveitis, choroiditis | 1/256 | 1/128 (62) | – |
| OO14† | 15/12 | F | Congenital toxoplasmosis (microcephalus, retarded development) | > 1/8,192 | > 1/16,384 ( > 8,000) | – |
| L109 | 34 | M | Asymptomatic latent | 1/8 | 1/16 ( < 7) | – |
| SK09 | 48 | M | Asymptomatic latent | 1/8 | 1/16 ( < 7) | – |
| FL14 | 58 | M | Asymptomatic latent | 1/8 | 1/64 ( < 7) | – |
| CH04 | 52 | M | Asymptomatic latent | 1/8 | 1/64 (31) | – |
| DF11 | 58 | M | Asymptomatic latent | 1/8 | 1/64 ( < 7) | – |

* Dye test titres were converted into iu/ml by comparison with the WHO reference standard (Lyng & Siim, 1982).

† OO14 is the congenitally infected daughter of OF14.

ND = not determined.

In the control group of 19 individuals, none had serological or clinical signs of *Toxoplasma* infection. The age range was between 19 and 36 years, and nine were male.

numbers. During the 4 day culture period, almost all host cells had been lysed by *Toxoplasma*; uninfected AGMPK cultures had overgrown and shed dead cells into the culture medium.

Patients were tested against a range of concentrations of each antigen (WLA, TSA and HCA). Optimum stimulation of lymphocytes from patients by WLA was in the same range as reported previously (Anderson *et al.*, 1979; Wilson *et al.*, 1980), i.e. between 12·5 and 6·25 μg/ml.

Optimum concentrations of TSA usually induced a ⩾ 10-fold increase in $^3$H-TdR incorporation when compared with unstimulated lymphocytes treated with native HCA. The majority of patients showed maximum incorporation of $^3$H-TdR with TSA at 50 μg/ml, with a clear decline in the dose–response curves thereafter (e.g. Fig. 2b). Seven patients showed maximum stimulation at the highest concentration tested (100 μg/ml; Fig. 2a). TSA was not tested at concentrations greater than

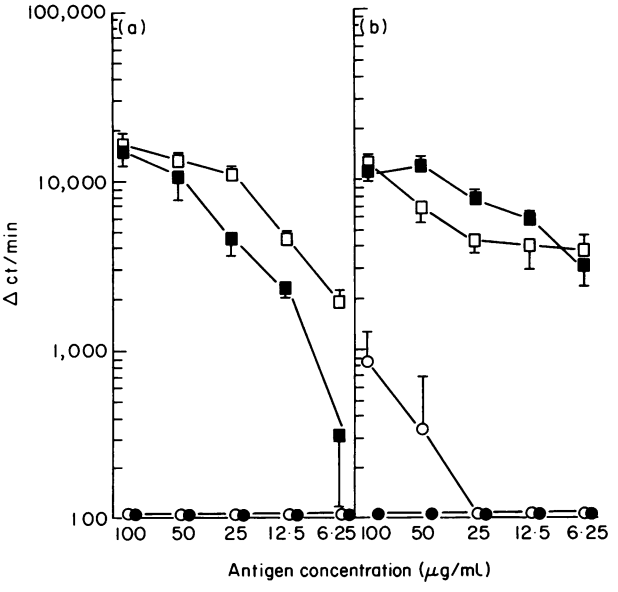


**Fig. 2.** Dose–response curves of lymphocyte stimulation by TSA or HCA. The results from two individuals are shown (a and b). ■ = mean Δ ct/min after incubation with native TSA; □ = mean Δ ct/min after incubation with heat treated (56°C for 30 min) TSA; ● = mean Δ ct/min after incubation with native HCA; ○ = mean ct/min after incubation with heat treated HCA. Error bars represent ± 1 s.d. from the mean of three determinations. Subject (a) showed an optimum response to TSA at 100 μg/ml; (b) showed an optimum response at 50 μg/ml. Subject (a) showed an enhanced response to heat treated TSA compared to native TSA, whereas the response of subject (b) was unaffected.

100 μg/ml as this would have increased both the relative concentrations of FBS and the toxic products released by dead or dying AGMPK cells.

The effect of prior heating of the TSA preparation to 56°C for 30 min was tested on the mitotic response of lymphocytes from five patients and was either without effect (Fig. 2b) or marginally increased mitogenesis (Fig. 2a). In only one instance did prior heating of HCA increase lymphocyte responsiveness at the higher concentrations tested (Fig. 2b).

Control (seronegative) individuals showed no significant lymphocyte stimulation with TSA or HCA, either in their native form or after heating.

The mean maximum $^3$H-TdR incorporation of lymphocytes from infected or control subjects stimulated with each of the three antigen preparations under test is summarized in Fig. 3. When lymphocytes were stimulated by the conventional *Toxoplasma* antigen, WLA (Fig. 3a), cells from infected patients showed a mean increase of $^3$H-TdR incorporation (Δ ct/min) of 21,800 ± 14,900 (mean ± s.d.) whereas control cells gave a Δ ct/min of 4,700 ± 17,600 (mean ± 3 s.d.). Although there was a significant difference between the mean responsiveness of the two study groups to this antigen, 50% of the infected patients were classed as negative responders as they were below the test threshold defined as the mean control Δ ct/min + 3 s.d.

In marked contrast, when lymphocytes were cultured with TSA (Fig. 3c), stimulation of $^3$H-TdR incorporation was uniformally positive (mean maximum Δ ct/min of 18,300 ± 12,000), due to the low incorporation by control lymphocytes cultured with TSA (347 ± 2,000; Δ ct/min ± 3 s.d.). Thus although TSA stimulation did not induce greater $^3$H-TdR incorporation than WLA in patients ($P > 0{\cdot}1$ by comparison of mean incorporation), its use provided a greater discrimination between infected and control groups, due to the low response of uninfected controls ($P < 0{\cdot}001$).

The response to the supernate of AGMPK cells, HCA, was low in both groups (Fig. 3b); infected patients gave a Δ ct/min of 480 ± 825 (mean ± s.d.) compared to a Δ ct/min of 205 ± 2,000 (mean ± 3 s.d.) for the control group.

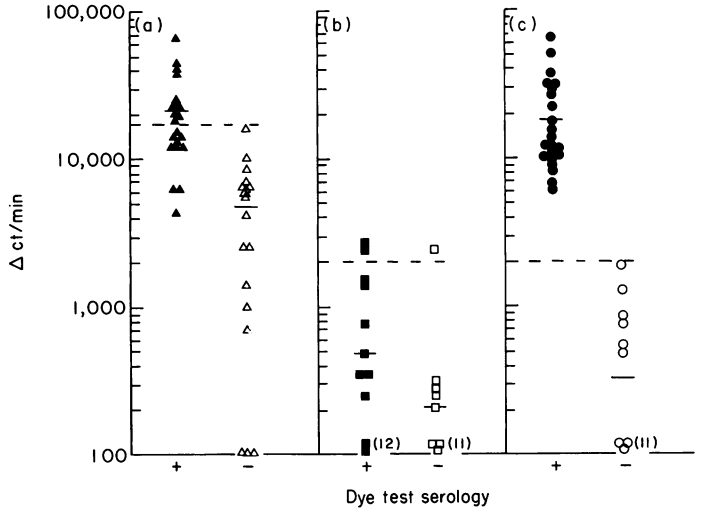


**Fig. 3.** Responses of infected (+) and normal (−) individuals to WLA, HCA and TSA (a, b and c, respectively), expressed as Δ ct/min. Short horizontal bars indicate the mean response of each group tested; broken lines, the threshold for positive transformation defined as the mean response +3 s.d. of lymphocytes from normal individuals cultured with each antigen preparation. Mean ct/min for lymphocytes cultured in the absence of antigen was 3,342±2,203 for infected individuals and 3,702±2,569 for normal individuals.

These experimental findings were re-analysed using stimulation index (SI) rather than increase in $^3$H-TdR incorporation, with virtually identical results (Fig. 4). Whereas 50% of infected patients failed to show a positive SI when stimulated with WLA, only one patient (5%) showed a low, negative SI with TSA.

Lymphocytes from 10 infected patients and seven controls showed a detectable increase in $^3$H-TdR incorporation after culture with HCA. The response of these individuals to HCA and TSA were compared in detail and is summarized in Fig. 5.

Infected patients showed a significant increase in mean responsiveness to HCA and TSA

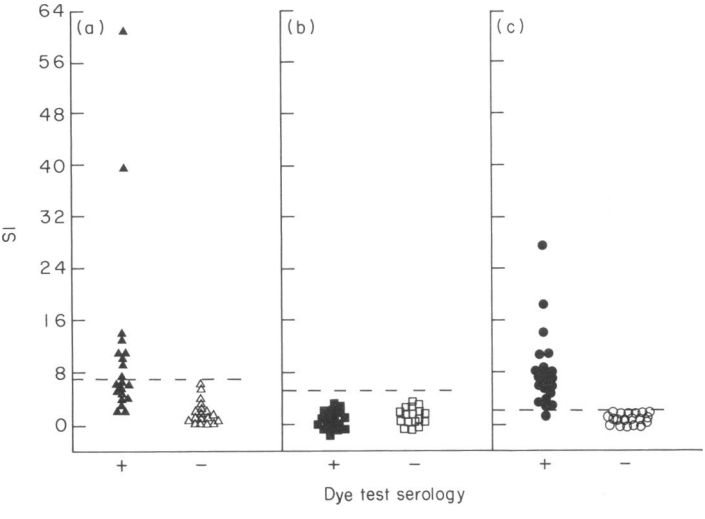


**Fig. 4.** Responses of infected (+) and normal (−) individuals to WLA, HCA and TSA (a, b and c, respectively), expressed as index of stimulation (SI). Horizontal broken lines represent the threshold for positive transformation, as defined in legend to Fig. 3.

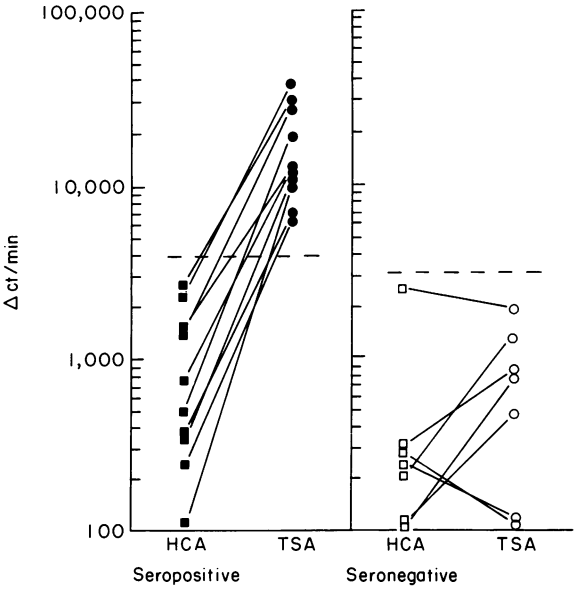


**Fig. 5.** Comparison of the response of normal (○, □) and infected individuals (●, ■) to HCA (□ ■) and TSA (○ ●). Horizontal broken lines represent the threshold for positive transformation as defined in legend to Fig. 3.

($P < 0{\cdot}001$), whereas there was no significant difference between the control responses ($P > 0{\cdot}1$). There was a net increase in $^3$H-TdR incorporation of approximately 1 order of magnitude when lymphocytes from patients were incubated with TSA compared with HCA (Fig. 5a). This relationship between the two preparations was stated as a null hypothesis ($H_0$) for regression analysis of the response to TSA against HCA for both infected and control subjects. $H_0$ was accepted by the regression of TSA against HCA for the infected patients, but not by the controls, indicating that there was only a net increase in ct/min in the patient group.

## DISCUSSION

Previous studies of lymphocyte transformation in toxoplasmosis have used soluble (predominently intracellular) antigens prepared by water lysis of *Toxoplasma* organisms (Hughes & Balfour, 1981; Johnson, McDonald & Neoh, 1981). During the course of infection, these intracellular components are probably not available to stimulate an immune response until the parasite has been killed, for example, by cell surface specific antibody and complement (Karim & Ludlam, 1975) opsinisation or direct phagocytosis (Hauser & Remington, 1981). In contrast, secretory antigens are continuously released and can be detected in plasma as early as 24 h post-infection (Van Knapen & Panggabean, 1977). Thus it is reasonable to expect that they might elicit an early immune response.

Secretory antigen (TSA) was directly compared with the standard water lysed *Toxoplasma* antigen that was prepared and used in the LTT as previously described (Anderson *et al.*, 1979). When results were expressed as ct/min, WLA was found to produce a 50% incidence of false negative results in seropositive individuals, in agreement with earlier studies on congenitally infected infants (Wilson *et al.*, 1980). This high incidence of negative results was not due to a low response by infected patients, but was due to the high mitogenic response to WLA shown by uninfected subjects (Fig. 3a & c).

In contrast to the response to WLA, there was a clear discrimination between TSA-induced blastogenesis in patients and controls (Fig. 3c). The increased specificity was not due to an enhanced response by the patients lymphocytes but a lower background response in the cultures of lymphocytes from seronegative controls ($P < 0{\cdot}001$, Fig. 3a & c). There was no significant difference between the mean patients' response to TSA compared to WLA. The difference between the responsiveness of control, seronegative patients cultured with WLA or TSA was highly significant

($P < 0{\cdot}001$, Fig 3a & c). In a minority of infected and control subjects, there was a low but detectable response to HCA, and so this was compared directly with the response to TSA (Fig. 5). Unlike controls, all patients showed a significant increase in responsiveness to TSA when compared with HCA. Previous studies on congenitally infected infants proposed that expression of results as stimulation indices (SI) was a more discriminating indicator of infection than increase in ct/min (Wilson *et al.*, 1980). In the present study this manipulation affected only the discrimination between control and patient responsiveness to TSA, which was less good when expressed as SI.

Isotope incorporation by lymphocytes cultured in medium alone showed a large deviation about the mean ($3{,}342 \pm 2{,}203$), which was probably due to the wide variety of signs and symptoms of the patient group compared with previous studies (Wilson *et al.*, 1980). Under these conditions therefore, expression of results as $\Delta$ ct/min appears to offer a more rational basis for the interpretation of results.

Antigens similar to WLA have been used extensively for the diagnosis of toxoplasmosis, either for LTT or indirect haemagglutination and enzyme linked immunosorbent assays. In the majority of cases, lysates have produced false negative results (Balfour, Bridges & Harford, 1980), and in some studies, a high frequency of false positives (Balsari *et al.*, 1980). Recently, Yano *et al.* (1983) described a water lysed preparation for LTT which they claimed was totally specific. Unfortunately, acutely infected patients were selected for this study using an indirect haemagglutination test, which is unreliable at this stage of the disease (Karim & Ludlam, 1975; Krahenbuhl & Remington, 1982), and the total number of patients studied was small. It seems therefore that the reliability of water lysed preparations for the detection of CMI by LTT is still open to doubt.

The reasons for the enhanced specificity of the LTT using TSA compared to WLA have not yet been fully established. The observation that the response of seronegative patients is lower to TSA than to WLA is probably due to a more restricted range or less mitogenic parasite antigens in the former preparation (Hughes, 1981).

The nature of TSA compared to previously defined secreted components is itself of interest. In contrast to other secretory components (Hughes & Balfour, 1981) prior heating of the antigen was found to either have little or no effect. In addition, TSA cannot be absorbed by the IgG fraction of a pool of human *Toxoplasma* antiserum (data not shown), indicating another basic difference to previously described secreted components (Hughes & Van Knapen, 1982).

Recent studies on secretions expressed by *Leishmania* during *in vitro* culture have shown that there is a suppression of lymphocyte transformation (Londner *et al.*, 1983). *Toxoplasma* secretions however are able to induce a strong proliferative response and preliminary studies on a T cell line established using TSA have indicated that it induces the rapid proliferation of an $OKT3^{+}4^{+}11^{+}8^{-}$ population of lymphocytes. The possible roles that TSA may have in the induction of cellular immunity, particularly macrophage activation, and its role in the pathogenesis of toxoplasmosis is currently under investigation.

The LTT for toxoplasmosis could play a potentially important role in the diagnosis of congenital infection. Serology has been shown to be ineffective in a number of cases, particularly those with a high incidence of false negative IgM responses (Wilson *et al.*, 1980). Diagnosis by biopsy and parasite isolation by subinoculation of tissue into experimental animals, is not only invasive, but may also require several weeks or months for a definitive diagnosis. In the immunosuppressed host, the LTT may be a useful indicator of immunity, as serological tests can be misleading, due to the persistence of high, rising antibody titres and the imminent danger of disseminated disease (Anderson *et al.*, 1983; McGregor *et al.*, 1984).

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