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Murine Model of Drug-induced Reactivation of *Toxoplasma gondii*

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Summary. A murine model of recrudescence of chronic toxoplasmosis, mimicking this life-threatening condition in immunosuppressed humans, was developed in Swiss-Webster mice infected 6 weeks previously with 10 cysts of the Me49 strain of *Toxoplasma gondii,* by treatment with dexamethasone (DXM, 2.5 mg/kgBM/day in drinking water), alone or combined with cortisone-acetate (CA, 50 mg/kg by subcutaneous injection 3 times a week). Treatment was continued for 7 weeks. Both DXM and DXM+CA treatment significantly increased mortality, as compared to infected untreated mice (P=0.0002 and P<10^{-4}, respectively), and to uninfected DXM-treated mice (P=0.043 and P=0.001, respectively). In both treatment groups, mean cyst numbers were 2-9-fold increased compared with chronically infected untreated mice. Spleen/body mass ratios and numbers of splenocytes were significantly lower (P<10^{-4}) than in untreated both infected and uninfected mice, indicating decreased immune reactivity in treated animals. Acquired immunity too was impaired by corticoids, as shown by lower resistance to challenge infection with the mouse-virulent RH strain of *T. gondii* in mice with established chronic infection treated with DXM than in those left untreated (P=0.038). Most importantly, 8 of the 56 treated animals (14.2%) developed clinical signs of toxoplasmic encephalitis, which was verified histologically. In these, survival was significantly shorter (P=0.009) and cyst numbers 6-fold augmented (P<10^{-4}) as compared to treated animals, which did not develop neurological signs. This simple model of drug-induced recrudescence of chronic toxoplasmosis, in addition to its potential use for *in vivo* studies of the pathogenic mechanisms of *T. gondii* reactivation, may be particularly suitable for the evaluation of chemotherapeutics.

Key words: chronic infection, corticoid drugs, immunosuppression, *Mus musculus,* reactivation, *Toxoplasma gondii*, toxoplasmosis.

Abbreviations: AIDS - acquired immunodeficiency syndrome, BM - body mass, HIV- human immunodeficiency virus, i.p. - intraperitoneal, s.c. - subcutaneous, TE - toxoplasmic encephalitis.

INTRODUCTION

Toxoplasma gondii is a ubiquitous intracellular protozoan parasite capable of infecting all types of nucleated mammalian host cells. Following a brief acute stage characterized by the proliferative tachyzoite stage of the parasite, primary *T. gondii* infection of an immunocompetent host is converted into latency, characterized by slowly growing bradyzoites within tissue cysts mostly found in the brain and skeletal muscles. Tissue cysts remain viable presumably for the life of the host, controlled mainly by cellular immune mechanisms (Hunter *et al.* 1996). However, if the balance between the host immune defenses and the parasite is disrupted, cyst rupture and renewed parasite proliferation may occur leading to clinical reactivation. The advent of AIDS and use of intensive immunosuppressive therapies for the

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treatment of malign and systemic diseases, or pre/post organ and tissue transplantation, have established a population of immunocompromised individuals prone to reactivation of opportunistic pathogens, including *T. gondii*, particularly in geographic areas with a high exposure to this protozoan. Interestingly, death due to toxoplasmosis following renal transplantation has recently been reported in pet animals as well (Bernsteen *et al.* 1999).

In patients with AIDS, *T. gondii* has emerged as the leading cause of focal cerebral lesions most commonly presenting as life-threatening TE (Luft and Remington 1992). Since current therapies are unable to eliminate the parasite from the infected host, treatment of a TE episode must be followed by life-long maintenance therapy. However, standard treatment is associated with considerable toxicity (Haverkos *et al.* 1987, Katlama 1996). Consequently, there is an urgent need for new therapeutics. We have shown in a murine model of acute toxoplasmosis that the combination of clindamycin and atovaquone may be promising (Djurković-Djaković *et al.* 1999); to further these studies an appropriate animal model is required, resembling the natural course of recrudescence of chronic toxoplasmosis in man.

While existing murine models have been invaluable for studying various aspects of the host-parasite relationship, many do not follow the natural history of *T. gondii* infection. A model of TE has been developed by direct intracerebral injection of *T. gondii* into mice immunosuppressed with cortisone acetate (Hofflin *et al.* 1987), in which TE is thus not a consequence of recrudescence of previously established infection. Dual infection models with *T. gondii* and a viral infection such as CMV (Pomeroy *et al.* 1989) and LP-BM5 MULv (Gazzinelli *et al.* 1992, Watanabe *et al.* 1993, Lacroix *et al.* 1994), have not had pharmacological applications due to their complexity and potential lack of reproducibility (Lacroix *et al.* 1996). In a SCID mouse model, rather than suppressing the host, sulfadiazine treatment suppressed infection thus allowing chronicity to develop, and its withdrawal led to relapse (Johnson 1992). Reactivation of the parasite was also attempted by administration of drugs with known immunosuppressive effect. Although Frenkel *et al.* (1975) have long ago induced reactivation of latent toxoplasmosis by cortisone in hamsters infected with the RH strain of *T. gondii,* in mice, however, Sumyuen *et al.* (1996) observed little reactivation following administration of drugs frequently used in organ transplantation, suggesting a mild immunosuppressive potential for cortisol acetate and azathioprine. On the other hand, Nicoll *et al.* (1997) obtained recrudescence with dexamethasone, but it is unclear why they failed to histologically demonstrate brain cysts in 60-70% of mice inoculated with *T. gondii*. Since corticosteroid drugs are often used in human therapy, we considered of interest to develop a murine model resembling the natural course of *T. gondii* reactivation in humans, in which consistently chronically infected immunocompetent mice are subsequently immunosuppressed with corticoid drugs.

MATERIALS AND METHODS

Mice. Female Swiss Webster mice (Medical Military Academy Animal Research Facility, Belgrade), 5-6 weeks old, weighing 18-22 g at the beginning of experiments were used. Mice were housed 6 per cage, and offered drinking water and regular mouse feed *ad libitum*, unless specified otherwise.

Parasites. Brain cysts of the Me49 strain of *T. gondii* (kindly provided by Dr. J. P. Dubey, Beltsville, MD), regularly maintained by passage through Swiss Webster mice twice a year, were used to establish chronic infection. The Me49 strain was used since it belongs to the type-2 strains, which account for 65% of cases of clinical toxoplasmosis in AIDS (Howe and Sibley 1995). To obtain brain cysts for experimental infections, mice infected at least 8 weeks previously were killed, brains removed and homogenized in a tissue homogenizer with 1 ml saline each. For cyst enumeration, 25 µl of the brain suspensions were placed on slides and microscopically counted. The number of cysts per brain was calculated by multiplying the number counted in 2 drops by 2 different investigators by 20, giving a threshold sensitivity of 20 cysts per brain. For experimental infections, mice were inoculated by intraoesophageal gavage with 250 µl brain suspensions assessed to contain 10 cysts. An inoculum of 10 cysts was chosen since in our experience an inoculum of this size regularly produces consistent infection without mortality, while larger inocula (>20) result in mortality unless treated with sulfadiazine.

For challenge experiments, tachyzoites of the virulent RH strain maintained through serial i.p. passages were used. For experimental infections, tachyzoites were harvested from mouse peritoneal fluids 72 h post-infection and purified by filtration through 3µm polycarbonate filters (Nuclepore ®, Maidstone, UK, lot 1050308). The parasites were counted in a haemocytometer and their numbers were adjusted to 2×10^6 / ml with saline. Suspensions were serially 10-fold diluted, and 0.5 ml aliquots of 2×10^2 / ml dilutions were inoculated i.p.

Drugs. Dexamethasone (DXM, dexamethasone sodium phosphate, ICN Galenika, Belgrade, Yugoslavia) was given at a dose of 2.5 mg/kgBM/day per mouse, obtained by dissolving 5 mg DXM per 1-liter drinking water. Treated as well as untreated water was changed 3 times a week.

Cortisone acetate (CA, hydrocortisone-21-acetate, lot 87494, ICN Biomedicals Inc., Aurora, OH) was administered by s.c. injection 3 times a week at 50 mg/kg BM.

Amoxicillin and clavulanic acid (Panklav®, Panfarma, Belgrade, Yugoslavia) was given in drinking water at 1g/l throughout the experiment to prevent bacterial superinfection.

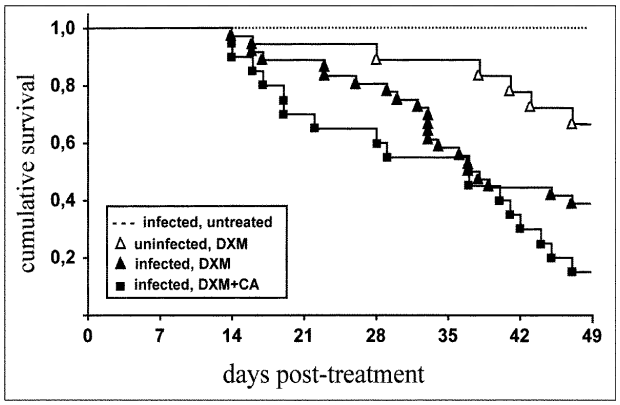

Fig. 1. Effect of DXM (2.5 mg/kg/day) alone or combined with CA (50 mg/kg/ 3 x week) on survival (Kaplan-Meier estimates) of mice with chronic *T. gondii* infection established by peroral inoculation of 10 Me49 cysts 6 weeks prior to treatment. Survival was significantly lower in infected DXM-treated mice than in infected untreated (P=0.0002) and uninfected DXM-treated mice (P=0.043), and in infected DXM+CA-treated mice than in infected untreated ($P<10^{-4}$) and uninfected DXM-treated mice (P=0.001)

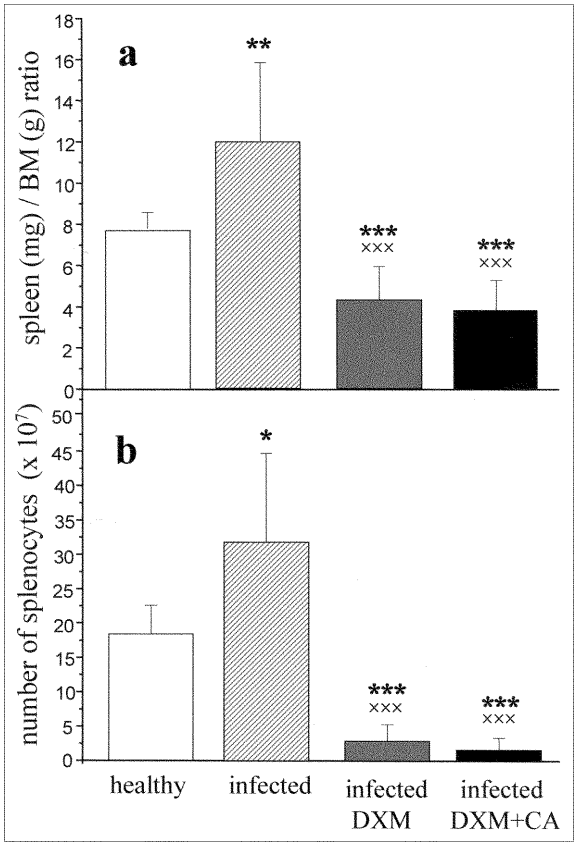

Fig. 2. a - effect of corticoid drug treatment of mice with established chronic *T. gondii* infection on spleen (mg) / BM (g) ratios; **b -** numbers of splenocytes. Data are presented as mean ± SD. Relative spleen size (** P=0.0004) and splenocyte numbers (* P=0.0072) were significantly greater in chronically infected untreated controls than in healthy control mice. In contrast, relative spleen size and number of splenocytes were significantly lower in both DXM and DXM+CA treated mice than in healthy (***$P<10^{-4}$) and chronically infected untreated (*** $P<10^{-4}$) controls

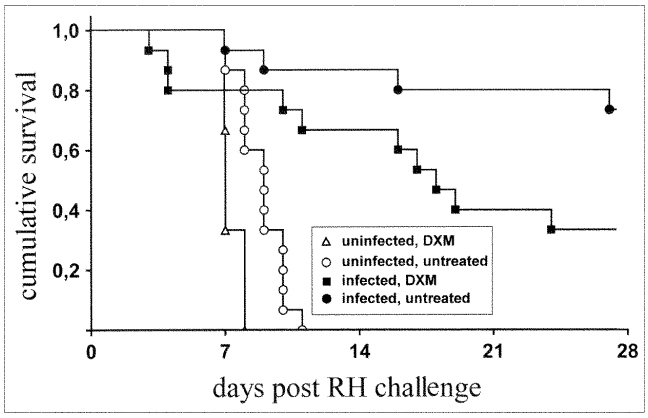

Fig. 3. Effect of i.p. challenge with 10^2 RH tachyzoites on survival of mice with previously established chronic *T. gondii* infection treated with DXM during 7 weeks. Survival times were calculated from day of RH challenge. Significantly lower survival is obvious in chronically infected DXM-treated mice than in chronically infected untreated mice (P=0.038), but longer than in uninfected untreated control mice (P=0.008)

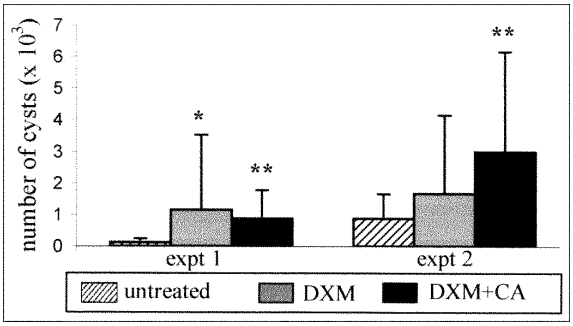

Fig. 4. Numbers of brain cysts (mean ± SD) in mice with established chronic toxoplasmosis after treatment with DXM alone or combined with CA. Compared to untreated chronically infected controls, cyst burdens were significantly higher in DXM-treated mice (* P=0.05) in experiment 1 and in DXM+CA-treated mice (** P=0.01) in either experiment

The immunosuppressive drug regimens were selected on the basis of preliminary experiments performed to assess the dosages of these drugs resulting in the highest degree of immunosuppression without causing lethality. For DXM, the assayed doses were 2.5 and 5 mg/kgBM/day (5 and 10 mg/l drinking water, respectively), similar to those used by Nicoll *et al.* (1997). Since in contrast to their study, the latter dose was toxic as shown by 80% mortality over a period of 6 weeks, the dose selected for this study was 2.5 mg/kg. For CA, the dose of 50 mg/kg was selected as well tolerated in both a similar study (Sumyuen *et al.* 1996) and our preliminary experiments. Since these had also revealed that CA did not cause sufficient reactivation as demonstrated by both little clinical signs and no mortality, the present study was designed on the basis of administration of DXM alone or combined with CA.

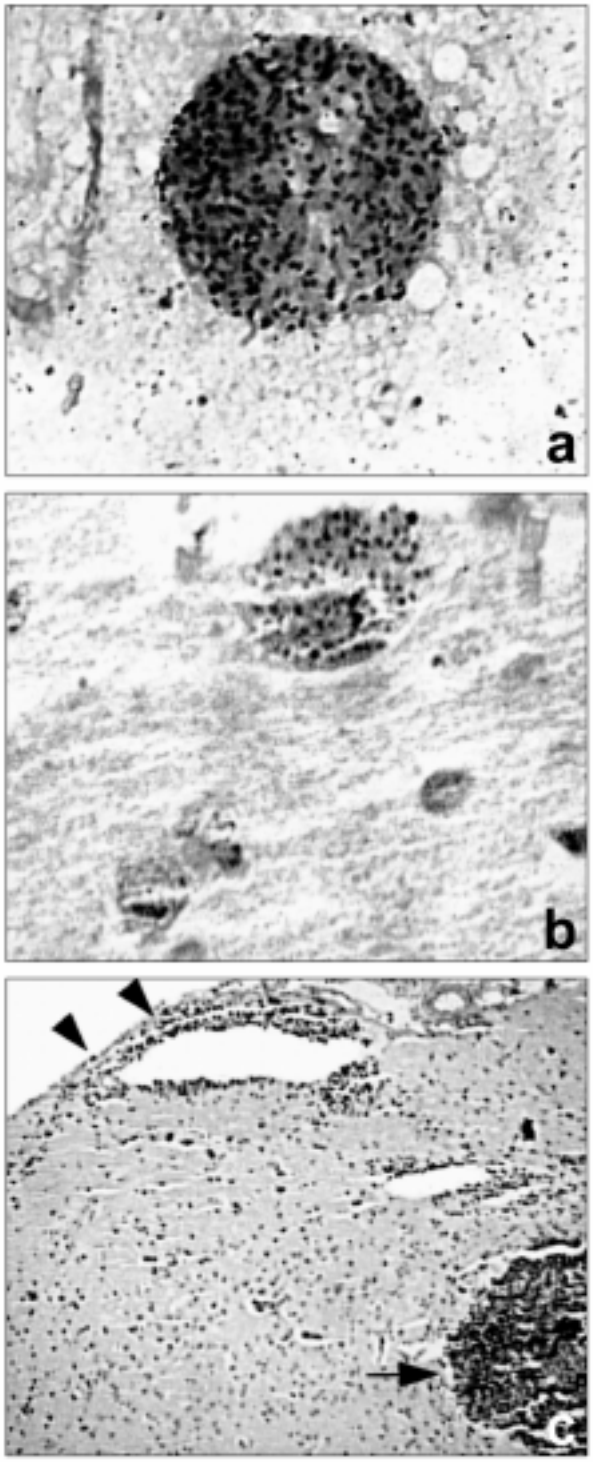

Fig. 5. Brain of mouse infected with 10 cysts of the Me49 strain of *T. gondii* 6 weeks prior to beginning of treatment with DXM (2.5mg/kg/day), which died with clinical signs of toxoplasmic encephalitis at day 32 day of treatment. Haematoxylin-eosin stain. **a** - squash smear of typical *T. gondii* tissue cyst. Magnification x 1000. **b -** brain section showing *T. gondii* tissue cyst with no inflammatory reaction in surrounding tissue. Magnification x 1000. **c -** brain section showing inflammatory infiltrate in meningeae and surrounding small blood vessels (arrowheads), and a large area of necrosis (arrow). Magnification x 200

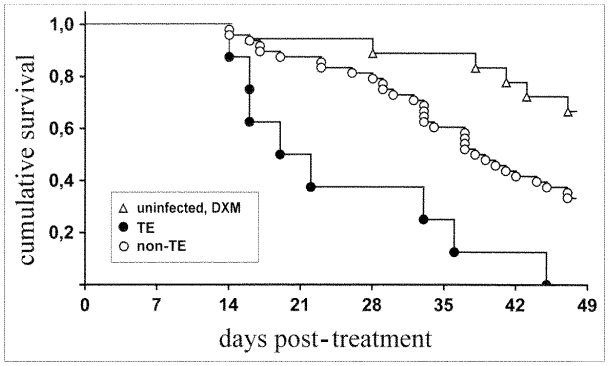

Fig. 6. Survival (Kaplan-Meier estimates) in mice with established chronic toxoplasmosis treated with corticoid drugs (both regimens) with respect to the occurrence of clinical TE. Survival was significantly lower in mice which developed TE than in those, which did not (P=0.009), but also lower in mice, which did not exhibit TE than in uninfected DXM-treated mice (P=0.01)

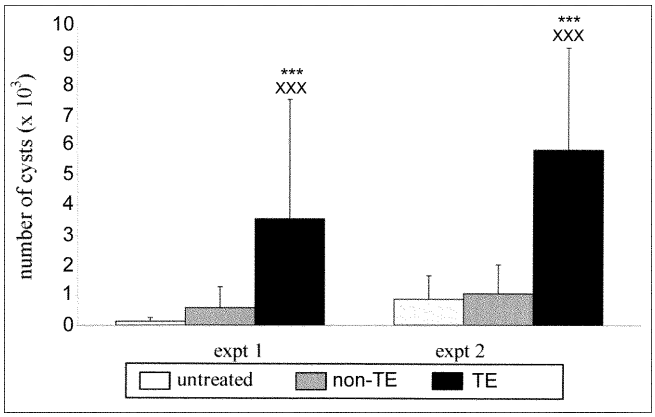

Fig. 7. Numbers of brain cysts (mean ± SD) in mice with established chronic toxoplasmosis treated with corticoid drugs with respect to the occurrence of clinical TE. Significantly higher cyst burdens were noted in mice which developed TE than in treated mice which did not exhibit neurological signs (***$P<10^{-4}$), and also than in chronically infected untreated mice (*** $P<10^{-4}$) in either experiment

Experimental Design. Groups of mice infected 6 weeks previously with cysts of the *T. gondii* Me49 strain were arbitrarily assigned to treatment with DXM alone or combined with CA over a period of 7 weeks, or left untreated as controls. Treatment groups comprised 6-12 animals each, depending on the experiment. Survival was monitored daily, and any clinical alterations (ruffled fur, hunchback appearance, locomotor alterations, paralysis) and deaths were recorded. Animals were weighed weekly. Mice dying during treatment were examined as described below. At the end of the 7-week treatment period, some survivors were challenged with RH strain parasites to examine the effect of corticoids on acquired immunity, and the remaining mice were killed by asphyxiation in chloroform for further examination. Each animal was weighed and brain and spleen

removed. Brain tissue homogenates were prepared for cyst enumeration. If histology was to be performed, brains were divided in halves; one half was homogenized, while the other was fixed in 9% formalin and paraffin-embedded, and 5 μm sections stained with haematoxylin-eosin. The relative spleen size determined as the spleen mass (mg)/ BM (g) ratio and total splenocyte numbers were both taken as parameters of immune activation / suppression. Thus, the spleens were weighed, and single cell suspensions made for enumeration of nucleated cells. The experiments were performed at least twice, and the results shown represent their cumulative results, unless specified otherwise.

Statistics

Survival of mice on different drug regimens was evaluated by the Kaplan-Meier product limit method. The difference between the curves obtained was analysed by the Peto and Peto Wilcoxon test. Differences in the cyst numbers, relative spleen sizes and total spleen cell counts between groups were examined by Student's t test. The level of statistical significance was 0.05.

RESULTS

Mice with established chronic toxoplasmosis were treated with DXM alone or combined with CA over a period of 7 weeks. Two control groups were included, comprising chronically infected untreated mice, and uninfected mice treated with DXM. All infected untreated mice survived. Treated animals, irrespective of whether infected or not, began exhibiting signs of general weakness and loss of subcutaneous adipose tissue as early as in the second week of treatment. In addition to general signs, some infected animals developed neurological signs compatible with the clinical presentation of TE. Mortality began after 2 weeks of treatment and continued throughout the treatment period. DXM and DXM+CA treatment resulted in 61.1% and 85% mortality, respectively. However, there was a 33% mortality in the control group of uninfected mice treated with DXM. Analysis of the estimated probabilities of survival is shown in Fig. 1. Both DXM and DXM+CA treatment significantly increased mortality, as compared to infected untreated mice ($P=0.0002$ and $P<10^{-4}$, respectively), and to uninfected DXM-treated mice ($P=0.043$ and $P=0.001$, respectively). Between the treatment groups, although DXM+CA treatment resulted in higher mortality, the difference in survival was not significant ($P>0.05$).

Evidence that corticoid drug treatment was actually immunosuppressive for treated mice was obtained by calculation of the spleen/BM ratios (Fig. 2a) and enumeration of spleen cells (Fig. 2b) at the time of death or at the end of the 7-week treatment period. Chronic infection was associated with significant spleen enlargement ($P=0.0004$) and marked increase in the number of splenocytes ($P=0.0072$) as compared to healthy controls. In contrast, in infected mice which received corticoids, both parameters were dramatically lower than in infected untreated mice ($P<10^{-4}$) and also than in healthy mice ($P<10^{-4}$). Between treatment groups, relative spleen size and cellularity were both lower, although insignificantly ($P>0.05$), in mice on the combined drugs than in mice treated with DXM alone.

To examine the effect of corticoid treatment on established protective immunity to *T. gondii*, groups of DXM-treated 7-week survivors were challenged with 10^2 tachyzoites of the RH strain of *T. gondii.* Infection with this strain, irrespective of the inoculum size, is invariably lethal for non-immunized mice. Groups of uninfected untreated mice, uninfected DXM-treated mice, and chronically infected untreated mice, were all included as controls. Uninfected untreated animals all succumbed to acute infection by day 11, as expected, and survival of uninfected treated mice was slightly lower ($P=0.052$) (Fig. 3). In contrast, mice with established chronic infection which received immunosuppressive treatment survived better than uninfected untreated controls ($P=0.008$), but less well than chronically infected untreated mice ($P=0.038$) of which, expectedly, most were protected against challenge. Evidence that these mice died of toxoplasmosis was provided by demonstration of masses of extracellular *T. gondii* tachyzoites in their peritoneal fluids.

In treated mice, the mean brain cysts counts were 2-9-fold increased as compared to those in chronically infected untreated mice killed at the end of the experiment (Fig. 4). However, the numbers of cysts in these chronically infected untreated controls varied greatly between different experiments (130 ± 122 in Expt 1 and 884 ± 748 in Expt 2). Thus, the results were analysed for each experiment separately. Compared to the cyst counts in untreated mice, despite the broad ranges in the numbers of cysts within each group, the increase was significant in mice on DXM treatment in the first ($P=0.05$), as well as in mice on DXM+CA treatment in either experiment ($P=0.013$ and $P=0.016$, respectively). It should be noted that in corticoid drug-treated mice, the cysts varied in size and quality; thus, there were large cysts containing numerous bradyzoites, as well as many small

cysts; some appeared normal while others were partly destroyed and/or degenerate. Similar as with other parameters, the numbers of cysts did not vary significantly between the treatment groups ($P>0.05$).

Most importantly, clinical reactivation manifested by decreased activity and tottering walk followed within days by chemiparesis and paralysis, developed, as mentioned above, in 8 of 56 (14.2%) mice on both treatment regimens. Evidence for these clinical observations was obtained by pathohistological analysis of brain sections of representative mice, which, in addition to brain cysts, showed perivascular and meningeal infiltration consistent with the diagnosis of meningoencephalitis (Fig. 5), despite the anti-inflammatory activity of corticoids. All animals which developed TE, died within days after the appearance of clinical signs, and their survival was significantly ($P=0.009$) shorter than that of treated mice without clinically evident TE (Fig. 6). On the other hand, treated mice not exhibiting neurological signs died more rapidly than uninfected DXM-treated mice ($P=0.01$). Furthermore, both spleen / BM ratios and splenocyte numbers in the TE mice (3.65 and $1.52x10^7$, respectively) were lower, although insignificantly ($P=0.32$ and $P=0.21$, respectively), than in mice which did not exhibit neurological signs (3.98 and $2.08x10^7$, respectively). Brain cyst burdens, both per experiment (Fig. 7) and cumulatively, were greatly (6-fold) increased compared with treated mice, which did not develop TE ($P<10^{-4}$), and (up to 27-fold) compared with chronically infected untreated mice ($P<10^{-4}$). The effect of the combined corticoid treatment was more rapid since mice on DXM+CA treatment exhibited clinical reactivation earlier during the course of treatment and died after a mean period of 18.3 days as compared to the mean survival of 29.2 days of mice treated with DXM alone.

DISCUSSION

The results presented demonstrate reactivation of previously latent murine *T. gondii* infection induced by 7-week-long administration of corticosteroid drugs DXM and CA. High mortality of treated animals was associated with increased brain cyst burdens and markedly lowered spleen size and cellularity, as compared to both healthy and infected untreated mice. Furthermore, clinical disease with typical locomotor signs of cerebral toxoplasmosis was demonstrated in 14.2% of the treated animals, associated with slightly deeper immunosuppression but significantly shorter survival and greatly increased (by 6-fold) brain cyst burdens compared with treated animals which did not exhibit TE. Since our work did not include a detailed histological study and histological sections were used merely for demonstration of encephalitis in evidently ill animals, it is possible that histologically demonstrable encephalitis existed in more treated animals. However, infected treated mice not displaying TE survived significantly shorter than uninfected treated mice, which suggests that neurological signs do not necessarily accompany death due to *T. gondii* reactivation. A parallel may be made with immunosuppressed humans, in which subclinical reactivation is more frequent than overt disease in transplant patients (Dérouin *et al.* 1987, Gallino *et al.* 1996).

The effect of corticoid drugs on acquired immunity in infected treated animals was shown by challenge with the RH strain of *T. gondii.* Between groups of mice with established chronic infection, a more detrimental effect of challenge infection in corticoid-treated mice than in those untreated, indicates impairment of protective immunity by corticoids. These findings are similar to those of Johnson *et al.* (1995), who showed that among mice immunized with a vaccine (ts-4) strain, RH challenge was less well tolerated by mice which received sublethal irradiation than by those which did not. Although we did not analyse the immunological mechanisms underlying impairment of acquired immunity by corticoids, the decrease in the numbers of CD4+ and CD8+ T cells shown to be induced by irradiation is plausible in our model too since general cellularity was greatly diminished.

The described model imitates the natural course of reactivation of *T. gondii* infection in man in that mice were infected with cysts of a type-2 strain responsible for most cases of human TE (Howe and Sibley 1995), using the peroral route which is the major route of human infection (Dubey and Beattie 1988). After the chronic infection was established, corticosteroid drugs often used in human therapy induced immunosuppression. Mice belong to a steroid sensitive species, and corticosteroids have long been known to cause destruction of murine lymphoid tissues, resulting in lymphocytopenia, monocytopenia, and shrinkage of the spleen (Antopol 1950). In addition, immunosuppression of mice by DXM has been reported to induce release of parasites from preexisting cysts and new cyst formation as early as after 6-12 days of administration (Odaert *et al.* 1996). Both regimens we used, DXM alone and combined with CA, induced significant immunosuppression and subse-

quent reactivation of the parasite in infected animals, but the effect of the combined treatment was greater, although not significantly, than the effect of DXM alone. While CA alone had just a mild effect, as shown by Sumuyen *et al.* (1996) and in our preliminary experiments, this study shows it potentiates the effect of DXM when the 2 are combined. However, it should be noted that a prolonged course of DXM itself causes considerable toxicity, since administration during 7 weeks resulted in death of one third of healthy control mice. These animals were progressively loosing weight and died with signs of general weakness. The discrepancy between our findings and those of Nicoll *et al.* (1997), who did not observe mortality with a prolonged administration of DXM even at a dose as high as 8 mg/L, is difficult to explain unless Swiss Webster mice are more susceptible to negative effects of DXM than the Porton mice used in their study.

The reproducibility of our model has been examined in repeated experiments yielding similar results. While parameters of immune activation varied little between experiments, the major difference was the baseline level of cysts formed in mouse brains following inoculation of cysts, obviously reflecting variations in the initial cyst content; this may vary between a few and several hundreds (Dubey 1997). However, such broad ranges are common in Swiss Webster mice after inoculation of even a controlled number of bradyzoites (Dubey 1997). On the other hand, the broad range of the numbers of cysts induced by treatment (wide standard deviations in Fig. 4) is easily explained since the data shown included all treated animals of which those that developed TE harbored dramatically greater cyst burdens. Hence, we chose to work with intact tissue cysts as the source of natural infection.

The presented model is simple to perform and to interpret since it does not include as many variables as coinfection models (Lacroix *et al.* 1996), and is not as requiring in terms of precautionary measures nor as costly as work with nude or SCID mice. In addition to the potential use of this model in studies of the pathogenic mechanisms of recrudescence of chronic toxoplasmosis, its simplicity and reproducibility recommend it particularly for the evaluation of chemotherapeutics, for which it is currently being used in our laboratory.

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