To investigate the role of cellular immunodeficiency in recurrent toxoplasmic retinochoroiditis, six Cynomolgus monkeys (Macaca fascicularis) with healed toxoplasmic lesions of the retina were immunosuppressed by total lymphoid irradiation. Three months prior to irradiation 30,000 Toxoplasma gondii organisms of the Beverley strain had been inoculated onto the macula of one eye in each monkey via a pars plana approach. Toxoplasmic retinochoroiditis developed in each animal, and lesions were allowed to heal without treatment. During total lymphoid irradiation animals received 2000 centigrays (cGy) over a 7-week period. Irradiation resulted in an immediate drop in total lymphocyte counts and decreased ability to stimulate lymphocytes by phytohemagglutinin. Weekly ophthalmoscopic examinations following irradiation failed to show evidence of recurrent ocular disease despite persistent immunodeficiency. Four months after irradiation live organisms were reinoculated onto the nasal retina of the same eye in each animal. Retinochoroidal lesions identical to those seen in primary disease developed in five of six animals. Toxoplasma organisms therefore were able to proliferate in ocular tissue following the administration of immunosuppressive therapy. This study fails to support the hypothesis that cellular immunodeficiency alone will initiate recurrent toxoplasmic retinochoroiditis. Results suggest that reactivation of disease from encysted organisms involves factors other than suppression of Toxoplasma proliferation. If reactivation occurs by other mechanisms, however, cellular immunodeficiency then may allow development of extensive disease. Invest Ophthalmol Vis Sci 29:835-842, 1988

Toxoplasmic retinochoroiditis in adults is usually a recurrent disease, arising at the borders of inactive retinochoroidal scars which are believed to be the residua of congenital Toxoplasma gondii infection.1 Primary ocular toxoplasmosis associated with acute systemic infection has been documented, but is uncommon in the United States.2 Extrapolation from experiments by Frenkel, who used the related parasite Besnoitia jellisoni, has led to the assumption that recurrent toxoplasmic retinochoroiditis results from the breakdown of encysted organisms within scars.3 Cyst breakdown could result in hypersensitivity reactions to exposed Toxoplasma antigens, or it could lead to a productive infection of adjacent retinal tissue by reactivated parasites. The relative importance of these potential disease mechanisms in the pathogenesis of recurrent ocular toxoplasmosis is not fully understood.

Studies using a nonhuman primate model of toxoplasmic retinochoroiditis indicate that hypersensitivity reactions alone are not responsible for the necrotizing retinitis of recurrent toxoplasmosis. Intravascular and intraocular injection of Toxoplasma antigens does not produce recurrent necrotizing retinochoroiditis in previously infected animals.4 Also, intraocular injection of live organisms or of Toxoplasma antigen does not produce necrotizing retinochoroiditis in previously immunized animals.5 It is assumed therefore that tissue invasion by live organ-
isms plays a major role in the pathogenesis of recurrent disease. This assumption is supported by autopsy cases showing that extensive retinal necrosis can be caused by *Toxoplasma* organisms in the absence of inflammatory cells.\(^6\)\(^7\) It remains unclear, however, what factors lead to the breakdown of cysts and the reactivation of parasites.

It has been well established that cell-mediated immunity plays a critical role in host defenses against intracellular parasites like *Toxoplasma gondii*.\(^8\)\(^9\) Severe *Toxoplasma* infections have frequently been associated with immunosuppressive therapy and diseases characterized by defects in cell-mediated immunity, including Hodgkin's disease and the acquired immunodeficiency syndrome (AIDS).\(^10\)\(^-\)\(^13\) Intracranial infections are the most frequent site of disease in such patients, but ocular toxoplasmosis has been reported as well.\(^6\)\(^7\)\(^10\)\(^-\)\(^18\) Toxoplasmosis may be due to newly acquired infection in immunosuppressed individuals, but reactivated disease is believed to be responsible for many of these cases.\(^11\)\(^13\) These observations have led to a hypothesis that cellular immunodeficiency may allow reactivation of organisms. To test the validity of this hypothesis with respect to ocular toxoplasmosis, nonhuman primates with healed toxoplasmic retinochoroidal lesions were immunosuppressed and observed for evidence of recurrent disease.

**Materials and Methods**

Five female and two male young adult Cynomolgus monkeys (*Macaca fascicularis*), weighing approximately 5 kg each, were studied. All seven animals were in good health, and all had normal retinas on indirect ophthalmoscopic examination prior to the start of these studies. All were Sabin-Feldman dye test-negative, indicating no prior exposure to *Toxoplasma gondii*.

The Beverley strain of *Toxoplasma gondii* was used. Parasites were maintained in mice by serial peritoneal injections of infected brain tissue. Parasitic inocula were prepared by diluting mouse peritoneal exudates with Hank’s solution containing 100 IU heparin sodium per 10 ml to a final concentration of 30,000 organisms per 0.01 ml. Organisms were harvested no earlier than 2 hr before inoculations into monkeys.

Prior to inoculation monkeys were anesthetized with a 2 ml intramuscular injection of a solution containing equal parts ketamine (100 mg/ml) and xylazine (20 mg/ml). Animals were placed in a supine position under the operating microscope and an O’Malley contact lens was placed over the left eye. Under direct visualization, a 25 gauge butterfly needle was passed through the pars plana, and the tip was placed directly onto the retina in the macular region. A 0.01 ml inoculum (30,000 organisms) was then injected through the needle. Monkeys were reinoculated by the same technique 4 months after immunosuppression. Organisms were placed over the nasal retina of the eye previously inoculated. After all inoculations, atropine 1% solution and neomycin-poly-mixin B-bacitracin ointment were placed in the eyes.

Viability of *Toxoplasma* organisms was confirmed after inoculations by injecting residual inocula into peritoneal cavities of mice. All mice developed clinical illness consistent with toxoplasmosis 7 to 9 days later, and the presence of organisms was confirmed by Giemsa staining of peritoneal fluid.

Eyes were examined by indirect ophthalmoscopy daily for 2 weeks following inoculation and weekly thereafter. Prior to examinations, animals received a 1 ml intramuscular injection of ketamine (100 mg/ml).

Three months after inoculation, one male monkey died of unrelated causes during the period of observation for evidence of disease reactivation. The inoculated eye was obtained immediately after death and placed in formalin for histologic examination.

**Total Lymphoid Irradiation**

Six monkeys were immunosuppressed by total lymphoid irradiation. Each animal received a total dose of 2000 centigrays (cGy) of lymphoid irradiation administered in 100 cGy fractions three times weekly over a 7 week period. The treatment field included all lymphoid regions extending from the superior cervical lymph nodes to the inguinal-femoral lymph nodes, including the spleen. Dosage was calculated at the midbody plane. Treatment was administered with 6 MeV photons produced on a linear accelerator at 100 cm source axis distance (SAD).

Prior to each treatment animals were anesthetized with a 1 ml intramuscular injection of ketamine. Animals were individually placed in a supine position under the linear accelerator and immobilized with tape. Custom cerrobend blocks were used to shield the lungs, liver and a portion of the bowel. Prior to administration of radiation, portal X-ray films were taken to verify treatment position.

**Parameters of Immunological Function**

Nonspecific tests of immune function were monitored. Blood was obtained from monkeys by venipuncture immediately before and at frequent intervals after total lymphoid irradiation. White blood counts and white blood cell differentials were used to calculate total lymphocyte counts.

A tritiated thymidine uptake assay measuring stimulation by phytohemagglutinin (PHA) of Ficoll-Hy-
Fig. 1. The border of a retinochoroidal scar following resolution of toxoplasmic retinochoroiditis contains a Toxoplasma gondii cyst (arrow) (hematoxylin, eosin ×540). Insert: The cyst is shown in higher magnification (×2550).

The assay was performed on lymphocytes obtained from five of six animals before irradiation, on all animals 1 month after irradiation, and on two animals 4 months after irradiation. All assays were performed in triplicate and average scintillation count values were recorded. A stimulation index was calculated for each assay by dividing scintillation counts of PHA-stimulated cells by scintillation counts of nonstimulated cells. Residual lymphocyte activity following immunosuppression was determined for five animals 1 month after completion of total lymphoid irradiation and for two animals 4 months after completion of total lymphoid irradiation. Values were calculated by dividing the postirradiation stimulation index by the preirradiation stimulation index and expressing it as a percentage.

This study conformed to the ARVO Resolution on the Use of Animals in Research.

Results

All animals developed positive Sabin-Feldman dye tests within 1 week of inoculation, indicating infection with Toxoplasma gondii. Each of the seven inoculated animals developed focal yellow-white retinochoroidal lesions at the site of inoculation. Animals also developed mild iritis, vitritis, and retinal vasculitis characterized by patchy white sheathing of the major arteriolar and venular arcades and their first branches. Retinochoroidal lesions enlarged over a 10-day period, then resolved during the following month, leaving a retinochoroidal scar with discrete borders. Vasculitis usually resolved over a 2- to 4-week period. Weekly examination by indirect ophthalmoscopy revealed no evidence of spontaneously recurrent retinochoroidal inflammation during a 2- to 3-month follow-up period after resolution of lesions.

The animal that died of unrelated causes had no clinical evidence of active toxoplasmosis at the time of death. Light microscopic examination of the inoculated eye revealed the presence of rare Toxoplasma gondii cysts (Fig. 1).

Animals tolerated total lymphoid irradiation well, with mild weight loss and transient diarrhea as the only observable clinical side effects. Serial examination by indirect ophthalmoscopy revealed no evi-
Total Lymphocyte Counts After Irradiation

Weeks After Start of Irradiation

Weeks After Start of Irradiation

Period of Irradiation

Fig. 2. Total circulating lymphocyte counts for six monkeys following total lymphoid irradiation. Symbols correspond to animal numbers in Table 1 as follows: open triangle, animal 1; closed circle, animal 2; open circle, animal 3; closed triangle, animal 4; closed square, animal 5; open square, animal 6.

dence of recurrent retinochoroiditis up to 4 months after the completion of radiation treatments.

Parameters of Immune Function

Total lymphoid irradiation resulted in an immediate drop in the number of total circulating lymphocytes (Fig. 2). Following irradiation, animals had decreased lymphocyte blastogenic response to PHA as measured by tritiated thymidine uptake assay (Table 1). Total lymphocyte counts generally reached their nadir during the fifth week of irradiation. Counts began to rise after completion of irradiation, approaching pretreatment levels by week 16. Two animals (Numbers 2 and 3 in Table 1) had low initial total lymphocyte counts. Both, however, had among the highest stimulation indices obtained on preirradiation testing (Table 1). Total lymphocyte counts generally reached their nadir during the fifth week of irradiation. Counts began to rise after completion of irradiation, approaching pretreatment levels by week 16. Two animals (Numbers 2 and 3 in Table 1) had low initial total lymphocyte counts. Both, however, had among the highest stimulation indices obtained on preirradiation testing, and in both, the stimulation indices fell with treatment. In one animal (Number 2), the low total lymphocyte count may have been artificial, since it was similar to those in other animals on subsequent testing and continued to fall with irradiation.

Despite the variability of preirradiation stimulation indices, each of five animals tested both before and after irradiation had a marked decrease in stimulation index 1 month after completion of treatment. Calculated values for residual lymphocyte activity ranged from 1 to 17% of preirradiation levels. Lymphocyte blastogenesis tested at 4 months after completion of irradiation in two animals revealed that residual lymphocyte activity remained decreased (6.1%, 30.9%), although total circulating lymphocyte counts had returned to normal.

Reinoculation of Organisms

Following reinoculation of organisms over the nasal retina after immunosuppression, five of six animals developed retinochoroidal lesions closely resembling the primary disease (Fig. 3). All animals, including the monkey that did not develop a retinochoroidal lesion (the surviving male), developed retinal vasculitis. In all animals vasculitis, anterior chamber reaction, and vitritis were less intense than they had been during the primary disease. Following reinoculation there was no change in the appearance of the macular retinochoroidal scars from the primary infection. All lesions resulting from reinoculation resolved over a 1-month period and left a retinochoroidal scar with distinct borders.

Discussion

Previous animal experimentation has provided conflicting data regarding the hypothesis that recurrent disease can be precipitated by immunodeficiency. Frenkel has shown that corticosteroid treatment and X-irradiation of hamsters infected with Toxoplasma results in exacerbation of systemic disease. O'Connor has induced recurrent ocular toxo-
plasmosis in rabbits by total body irradiation.\textsuperscript{21} Kaufman has shown that rabbits with old, experimentally-induced ocular Toxoplasma infections may succumb to systemic disease when treated with corticosteroids, but he was unable to produce reactivation of toxoplasmic retinochoroiditis by such treatment.\textsuperscript{22}

To pursue the study of recurrent ocular disease, a nonhuman primate model was chosen.\textsuperscript{23} Because of its lethal susceptibility to Toxoplasma gondii infection, the rabbit was felt to be an inadequate model for further study of ocular toxoplasmosis. Also, there are significant differences between rabbits and humans in ocular anatomy and immune responses.

Following resolution of their primary disease, all monkeys developed retinochoroidal scars similar to those of toxoplasmic disease in humans. The Beverley strain of organisms was selected for these experiments because of its known tendency to form cysts in tissue.\textsuperscript{24} The fact that cysts were found in healed retinal lesions on histologic examination of one animal indicates that this nonhuman primate model may be appropriate for the study of factors pertinent to the reactivation of human ocular disease.

Total lymphoid irradiation was chosen to immunosuppress animals because of its relatively selective effect on cell-mediated immunity.\textsuperscript{25-28} Also it is easily administered and its effects are long-lasting. Because the head can be shielded during irradiation, total lymphoid irradiation has a theoretical advantage over other immunosuppressive therapies in the study of recurrent disease. There can be no direct effect on encysted organisms, as might occur with the systemic administration of immunosuppressive drugs.

Specific immunological response to Toxoplasma

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**Table 1. Preirradiation and postirradiation lymphocyte stimulation assays**

<table>
<thead>
<tr>
<th>Animal preirradiation</th>
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\* All tests performed in triplicate. Values indicate mean counts.
\*\* NS = scintillation counts (CPM) of nonstimulated cells.
\*\* SI = stimulation index (S/NS).
\*\* RLA = residual lymphocyte activity [(postirradiation SI/preirradiation SI) \times 100].

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Fig. 3. Fundus montage of the left eye in a monkey 1 week after re inoculation of organisms over the nasal retina. The nasal lesion (black arrows) has the "soft" appearance of active inflammation (which also was characteristic of the primary infection), in contrast to the "hard" appearance of the macular retinochoroidal scar in the site of the primary infection (white arrow).
antigen was not determined. In prior experiments at the Francis I. Proctor Foundation using infected and immunized Cynomolgus monkeys, consistent, reproducible measures of lymphocyte blastogenic response to Toxoplasma antigens could not be achieved (unpublished data), thus limiting the value of this test for the current studies. Nonspecific parameters of immune function, however, including decreased total lymphocyte count and decreased blastogenic response to PHA, were consistent with the cellular immunosuppression following total lymphoid irradiation reported by other investigators. The return of total lymphocyte counts to pretreatment levels following cessation of radiation treatments does not necessarily indicate a return of immunocompetence. Allograft tolerance in total lymphoid irradiated baboons is not a function of reduced lymphocyte numbers. Total lymphoid irradiation can result in B-lymphocytosis and T-lymphopenia. Cellular immune function therefore can remain depressed despite quantitatively normal lymphocyte counts. The persistence of suppressed lymphocyte blastogenic response to PHA was confirmed in two animals 4 months after radiation treatments ended.

Despite evidence of immunosuppression, animals did not develop recurrent ocular toxoplasmosis. If cellular immunodeficiency alone were to cause reactivation of infection, it would be expected to occur during the early postirradiation period, when immunosuppression is at its maximum. These studies therefore fail to support the hypothesis that cellular immunodeficiency results in development of recurrent ocular disease. They suggest that defects in cell-mediated immunity are insufficient stimuli for reactivation of encysted organisms in healed retinal scars.

Evidence from clinical reports also support this conclusion. Despite the severe defects in cellular immunity that occur in patients with AIDS, toxoplasmic retinochoroidal scars may be seen in these patients without evidence of reactivation. Incidental Toxoplasma cysts have been seen in brain tissue of an AIDS patient without signs of active disease. Although toxoplasmosis is the most common nonviral intracranial infection in patients with AIDS, ocular toxoplasmosis is uncommon. When it does occur, ocular infections are not seen adjacent to scars, which suggests that these ocular lesions may be sites of new infection rather than reactivated disease.

Several potential sources of error in these experiments could have contributed to the lack of disease reactivation in monkeys. Although typical Toxoplasma cysts were found in one animal after infection, their presence was not confirmed in other animals. Neither was the viability of cysts determined. Also the Cynomolgus species appears to be relatively resistant to Toxoplasma infection. Finally, the monkeys may have been inadequately immunosuppressed. They may have retained enough cellular immune function to prevent proliferation of organisms even if cyst breakdown occurred.

To test the latter possibility, animals were reinoculated after total lymphoid irradiation with live organisms in a different portion of the retina by the same technique. New lesions would not be expected in immunocompetent animals. In immunocompetent rabbits, repeat inoculation of live organisms results only in an attenuated form of disease. Monkeys immunized by Toxoplasma antigen do not develop active foci of infection following intraocular inoculation of organisms. Five of six monkeys in this experiment developed new lesions similar to their primary disease. Organisms therefore were able to proliferate in the eye after the immunosuppressive therapy had been administered. The lack of recurrent disease in the original experiment could not be attributed simply to residual immunity that suppressed the proliferation of live parasites.

It is most likely that reactivation of encysted organisms and recurrent ocular toxoplasmosis are attributable to multiple factors. Immunologic factors other than cell-mediated immunity may play a role. The presence of vasculitis after reinoculation, which has been attributed in other studies to immune complex formation, indicates functional humoral immune reactions in these animals. High levels of Toxoplasma antibodies, which can be seen in patients with cellular immune defects, are not protective. Antibodies are believed to be important stimuli for cyst formation, however. Elevated levels of antibodies therefore may favor the retention of intact cysts in the retina as well.

Although their assertions were never proven, various investigators have hypothesized that cysts spontaneously break down over time, releasing viable organisms. Electron microscopic studies have shown evidence of cyst degeneration in toxoplasmic retinochoroidal lesions. Cyst walls may also be degraded by enzymes released from inflammatory cells in adjacent tissue. Patients with a history of toxoplasmic retinochoroiditis are known to have circulating anti-retinal antibodies, which could be one source of nontoxoplasmic inflammation capable of reactivating encysted organisms.

Ocular toxoplasmosis has been seen in eyes following trauma. It has been suggested that trauma is a means of disrupting Toxoplasma cysts mechanically and of releasing viable organisms. Trauma alone, however, does not result in reactivation of disease in
an experimental model, even when blunt force sufficient to cause retinal disorganization is applied to the highly susceptible rabbit as host.32,39

Other, as yet unidentified, factors also may be related to reactivation of parasites. It is likely that release of viable organisms from cysts requires one or more mechanisms unrelated to the host's immune response. Parasitic factors and the host's immune status then may determine the extent of Toxoplasma proliferation and recurrent retinochoroiditis. The virulence of organisms varies between different strains of Toxoplasma.40 Some may be able to proliferate transiently even in the face of clinically normal immune function. When encysted organisms are released, or newly acquired organisms disseminate to the eye, in a setting of disturbed immune function (somewhat analogous to the experiments reported here), proliferation may be facilitated and severe ocular disease may ensue.

Key words: immunosuppression, nonhuman primate, retinochoroiditis, total lymphoid irradiation, Toxoplasma gondii

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References


