Inoculation of herpes simplex virus type 1 (HSV-1; KOS strain) into one anterior chamber of euthymic BALB/c mice results in virus infection of the contralateral optic nerve and retina in about 90% of infected mice beginning on day 7 postinoculation (p.i.). The peak of virus replication in the retina of the uninoculated eye has been divided into three phases: acute retinitis, retinal necrosis, and resolution. Acute retinitis is observed between days 7 and 9 p.i. and the titer of infectious virus declines rapidly after this time. The median virus titer in the uninjected eye of euthymic mice at day 14 is less than 100 plaque-forming units (PFU), and all virus is cleared from the uninjected eye by day 21.

The infectious process in the retina of the uninoculated eye has been divided into three phases: acute retinitis, retinal necrosis, and resolution. Acute retinitis is observed between days 7 and 9 p.i. Because T cells are not observed in the contralateral retina at day 10 p.i. and depletion of CD4+ or CD8+ T cells does not affect the severity of retinitis or the titer of virus in the contralateral eye at day 9 p.i., acute retinitis is caused primarily by virus replication in the retina of the uninjected eye. Retinal necrosis begins on day 10 p.i. and appears to require the participation of CD8+ T cells. Results from T cell depletion studies demonstrated that the severity of retinal infection was significantly reduced and virus clearance was delayed at day 14 p.i. only in CD4-depleted mice. The resolution phase begins on or about day 15 p.i. Early in this phase, all remaining replicating virus is cleared from the uninjected eye. Later in this phase, the remnants of the retina are organized into a fibrocellular scar, and the ocular inflammation is gradually resolved.

Based on the results of previous studies, it is hypothesized that T cells are recruited to the infected contralateral eye at or near the time of acute retinal necrosis. To test this hypothesis, immunohistochemistry and flow cytometry were used to determine the pattern of T cell infiltration into the uninoculated contralateral eye as well as the percentage of T cells in the contralateral eye during all phases of the infection.

METHODS

Mice

Adult (≥6 weeks) euthymic female BALB/c mice (Ia+) were purchased from Taconic (Germantown, NY). Mice were housed in accordance with National Institutes of Health Guidelines, and all animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sodium pentobarbital (0.65 mg/10 g body weight) was used as the anesthetic agent for all ocular inoculations and for clinical observations.
**Virus**

The KOS strain of HSV-1 was used in these experiments. Virus stocks were prepared, aliquoted, and stored at −70°C as described previously. The titer of stock virus was determined by plaque assay on Vero cells and was 2.5 × 10^8 PFU/ml. For each experiment, a fresh aliquot of stock virus was thawed and diluted to the appropriate concentration immediately before ocular inoculation.

**Immunohistochemistry**

Mice were killed and perfused with phosphate-buffered saline (PBS). The spleens and contralateral eyes were removed, embedded in OCT compound (Miles, Elkhart, IN), and sectioned at 8 μm using a cryostat. All sections were dried in air and kept at 4°C overnight before staining at room temperature. Sections were fixed in acetone (5 minutes) and incubated with 3% (vol/vol) normal goat serum (15 minutes). Excess serum was blotted, and the sections were incubated for 45 minutes with biotinylated rat monoclonal antibodies (mAbs) specific for mouse CD4^+^ T cells or CD8^+^ T cells (Life Technologies, Gaithersburg, MD). All mAbs were diluted with 3% (w/v) bovine serum albumin in PBS (bovine serum albumin solution) to a concentration of 2.5 to 5.0 μg/ml. The sections were washed twice with PBS and placed in 0.5% (vol/vol) hydrogen peroxide in methanol for 20 minutes. The sections were washed once with PBS and incubated with avidin-biotinylated ABC enzyme complex reagent (Vector Laboratories, Burlingame, CA) for 30 minutes. The sections were then washed three times with PBS and 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Chemical, St. Louis, MO) supplemented with 0.3% (wt/vol) nickel chloride and 0.01% (vol/vol) hydrogen peroxide was added. Color development was monitored microscopically. All sections were counterstained with hematoxylin and examined microscopically.

**Cell Collection and Flow Cytometry**

Mice were killed and perfused with PBS, and the contralateral eyes and contralateral superficial cervical lymph nodes were collected. Lymph node cells were collected by placing the lymph nodes on 100-μm nylon mesh and teasing the tissue gently with forceps. The cell suspension was washed twice with PBS and resuspended in PBS. For ocular cell collection, the conjunctiva and extracocular muscles were removed from the enucleated eye. To minimize cell loss, the eye was positioned on a 1-cm² nylon mesh square placed over the opening of a 1.5-ml microcentrifuge tube filled with complete Dulbecco’s modified Eagle’s medium with 5% serum. The lens, iris, and ciliary body were removed. The retina and the choroid were dissected from the posterior segment, and the posterior uveal tract and the sensory retina were carefully teased on the nylon mesh until only fibrous tissue remained. The cell suspension was pelleted and resuspended in PBS. After resuspension, aliquots of each sample were transferred to fresh microcentrifuge tubes. The cells were repelleted, the supernatant was aspirated, and the cells were resuspended in the residual PBS and incubated for 30 minutes on ice with 5 μl of fluorescein isothiocyanate-conjugated rat anti-mouse CD4 mAb (Life Technologies), 5 μl of fluorescein isothiocyanate-conjugated rat anti-mouse CD8 mAb (Becton-Dickinson, Mountain View, CA), or 5 μl of bovine serum albumin solution. The monoclonal antibodies were diluted with bovine serum albumin solution; the concentration of the anti-CD4 mAb was 0.5 mg/ml, and the concentration of the anti-CD8 mAb was 0.2 mg/ml. Cells were washed once with PBS containing 50 μg/ml propidium iodide (PI; Sigma Chemical) resuspended in 0.25 ml to 0.5 ml of PBS with 5% (vol/vol) fetal calf serum, and passed through nylon mesh before flow cytometry.

Flow cytometry was done on a FACStar plus (Becton Dickinson, Mountain View, CA) using a single excitation wavelength of 488 nm. A gate to select mononuclear cells and to exclude dead cells and debris was set at the time of analysis using a single cell suspension stained with PI alone. To verify specificity of gating, cells from a normal lymph node, from a normal eye, and from an uninoculated eye with HSV-1 retinitis at day 11 p.i. were isolated, stained for CD4^+^ and CD8^+^ T cells, and analyzed (Figs. 1A through IF). As an additional control, at day 11 p.i., cells from the uninoculated eye of a CD4-depleted mouse with retinitis stained for CD4^+^ and CD8^+^, and cells from the uninoculated eye of a CD8-depleted mouse with retinitis stained for CD4^+^ and CD8^+^ (Figs. 1G through 1J). The dotted line indicates unstained cells, and the solid line indicates stained cells.

**T Cells in the Uninjected Eye**

![Flow cytometry results demonstrating gate selection to detect CD4^+^ and CD8^+^ T cells isolated from eyes with HSV-1 retinitis.](image-url)

Cells from a normal lymph node stained for CD4 (A) and for CD8 (B), cells from a normal eye of an uninfected mouse stained for CD4 (C) and for CD8 (D), cells from an uninoculated eye with HSV-1 retinitis at day 11 p.i. stained for CD4 (E) and CD8 (F), cells from the uninoculated eye of a CD4-depleted mouse with retinitis stained for CD4 (G) and CD8 (H), and cells from the uninoculated eye of a CD8-depleted mouse with retinitis stained for CD4 (I) and CD8 (J). The dotted line indicates unstained cells, and the solid line indicates stained cells.
RESULTS

Immunohistochemistry

Initial studies were done to verify the ability of the anti-CD4 and anti-CD8 mAbs to detect T cells in the spleen of a normal uninfected mouse (Figs. 2A, 2B). As shown in Figure 2C, an adjacent section of the spleen did not react with an isotype-matched rat anti-mouse Iaα (Life Technologies). In addition, the retina of a normal eye from an uninfected mouse did not contain either CD4+ or CD8+ T cells (Figs. 2D, 2E).

For these experiments, adjacent serial sections from mice with clinical evidence of retinitis at day 8 p.i. were stained with anti-CD4 or anti-CD8 mAb from day 9 p.i. through day 63 p.i. At day 9 p.i., CD4+ and CD8+ T cells were observed in the iris and ciliary body (not shown) and in the choroid (Figs. 3A, 3B). T cells are not seen in the sensory retina even though the retina is infected with virus and became disorganized at that time.11 At day 11 p.i., T cells were observed in the sensory retina, and the number of T cells in the choroid appeared to be increasing (Figs. 3C, 3D). By day 14 p.i., fewer T cells were observed in the choroid, whereas the number of CD4+ and CD8+ T cells in the retina still appeared to be increasing (Figs. 3E, 3F). By day 21 p.i., the border between the sensory retina and the choroid became indistinct, and the retina began to be replaced by fibrous tissue (Figs. 4A, and 4B). At that time, the maximum number of T cells was observed in the posterior segment. By day 35 p.i., fibrous tissue replacement of the retina continued, and the number of CD4+ and CD8+ T cells was decreased (Figs. 4C, 4D). Also, at that time, the un.injected eye was becoming phthisical (not shown). By day 63 p.i., the retina was completely replaced by fibrous tissue that contained both CD4+ and CD8+ T cells within its interstices (Figs. 4E, 4F).

Flow Cytometry

As a result of the extent of retinal destruction and difficulty in selecting representative sections that were equivalent from eye to eye at all time points, it was not possible to quantify the number of T cells in each eye using histopathologic methods. Therefore, flow cytometry was used to confirm the histopathologic observations and to determine the percentage of CD4+ and CD8+ T cells in the uninoculated eye during all phases of the infectious process. Similar to the histopathologic observations, flow cytometry revealed an increasing number of CD4+ and CD8+ T cells from day 9 p.i. through day 21 p.i. with a decreasing number of CD4+ and CD8+ T cells on day 35 p.i. and day 63 p.i. (Fig. 5). The average percentages of CD4+ T cells were 5.6%, 16.4%, 23.0%, 36.1%, 18.2%, and 5.2% and of CD8+ T cells were 6.6%, 14.0%, 27.7%, 35.9%, 31.7%, and 12.0% at days 9, 11, 14, 21, 35, and 63 p.i., respectively. The maximum percentage of both CD4+ and CD8+ T cells was observed at day 21 p.i. The percentage of CD4+ and CD8+ T cells at day 21 p.i. in the un injected eye of a mouse that did not develop retinitis was 0.7% and 0.5%, respectively (not shown). These results provide flow cytometric confirmation of the histopathologic findings shown in Figures 2D and 2E. In contrast to the flow cytometry results in the uninoculated eye, the percentage of CD4+ and CD8+ T cells in the draining lymph nodes from HSV-1-infected mice remained relatively constant during the course of the experiment (Fig. 5).

DISCUSSION

After unilateral anterior chamber inoculation of the KOS strain of HSV-1, the retina of the uninoculated eye becomes infected with virus, and acute retinitis is observed in the uninoculated eye beginning on day 7 p.i.6,11 Acute retinitis is followed by retinal necrosis, and retinal necrosis is followed by resolution, during which the inflammatory process in the uninoculated eye is resolved and the remnants of the retina become organized into a fibrocellular scar.6 Although an unequivocal role for T cells in protection of the retina of the injected eye has been demonstrated,2,12-14 the role of T cells in the pathogen-
FIGURE 3. Retinal cross-sections illustrating recruitment of T cells (dark-stained cells at arrow tips) to the contralateral uvea and retina after anterior chamber inoculation of HSV-1. (a) CD4+ cells, day 9 p.i.; (b) CD8+ cells, day 9 p.i.; (c) CD4+ cells, day 11 p.i.; (d) CD8+ cells, day 11 p.i.; (e) CD4+ cells, day 14 p.i.; and (f) CD8+ cells, day 14 p.i. The choroid is indicated by an asterisk. Original magnification: 132X.

FIGURE 4. Retinal cross sections illustrating persistence of T cells (dark-stained cells at arrow tips) in the posterior segment of the contralateral eye during the resolution phase after anterior chamber inoculation of HSV-1. (a) CD4+ cells, day 21 p.i.; (b) CD8+ cells, day 21 p.i.; (c) CD4+ cells, day 35 p.i.; (d) CD8+ cells, day 35 p.i.; (e) CD4+ cells, day 63 p.i.; and (f) CD8+ cells, day 63 p.i. The area of the fibrocellular scar is indicated by a bracket in C-F. Original magnification: 132X.
Figure 5. Flow cytometry results after uniocular anterior chamber inoculation of HSV-1 from day 9 p.i. through day 63 p.i. (A–F). Each closed circle represents the percentage of CD4+ and CD8+ cells in a single eye with retinitis, and each open circle represents the percentage of CD4+ and CD8+ cells in the draining lymph nodes from a mouse with retinitis.

CD4+ T cells are involved in the transition from acute retinitis to retinal necrosis. Together, the results of these two previous studies suggest that examination of later time points is needed. Therefore, the present study used both immunohistochemistry and flow cytometry to examine uninoculated eyes from day 9 p.i. until day 63 p.i. These new studies confirm early (by day 9 p.i.) recruitment of T cells to the choroid of the uninoculated eye and provide additional evidence in support of the idea that T cells are more important during the necrosis and resolution phases of the disease than during early, acute retinitis.

In conclusion, these studies support the hypothesis that T cells are involved in the later stages (necrosis and resolution) of HSV-1 infection of the retina of the uninoculated eye after uniocular anterior chamber inoculation of virus. Studies are now in progress to define the mechanism(s) by which CD4+ T cells and, perhaps, CD8+ T cells contribute to this disease process.
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References