Experimental Corneal Endotheliitis in Rabbit

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PURPOSE. Corneal endotheliitis may cause permanent visual loss due to endothelial decompensation. The pathogenesis underlying this distinct clinical entity is not known. In the current study, a rabbit herpetic corneal endotheliitis model was made of induced anterior chamber-associated immune deviation (ACAID).

METHODS. One group of rabbits received left-eye intracameral inoculation of UV-inactivated herpes simplex virus (HSV)-1 (strain McKrae). The second group received cell medium in the same manner as the first group. The third group subcutaneously received the same inoculum as the first group. Seven days later, all right eyes were intracameraly infected with 2.5 \times 10^4 plaque-forming units of infectious HSV-1. Eyes were evaluated by slit lamp examination. Two weeks after infection, rabbits were killed, and right eyes were examined by immunohistochemical staining and electron microscopy. Aqueous humor was detected for HSV-1 DNA and antibody.

RESULTS. Nonspecific inflammation occurred in the anterior segments of the eyes from the second and third groups. In contrast, at 14 days after infection, the first group of rabbits showed a specific pattern of inflammation that greatly resembled clinical features of corneal endotheliitis. Viral antigen was detected only in the endothelial layer. Electron microscopy revealed enlarged intercellular gaps and infiltration of inflammatory cells that are characteristic of endothelial defects. HSV-1 DNA was detected at a significantly higher number in the aqueous humor aspirates from endotheliitis rabbits. In addition, ACAID was shown to be induced in the rabbits with corneal endotheliitis.

CONCLUSIONS. HSV-1 infection can induce corneal endotheliitis and ACAID may play the pivotal role in this entity. (Invest Ophthalmol Vis Sci. 2000;41:377–385)

Herpes simplex virus (HSV) infection is the leading cause of infectious corneal disease in the world. It has been generally accepted that herpetic epithelial keratitis results directly from viral replication in epithelial cells, and stromal keratitis is assumed to result from a host immune response to viral antigen present in the stroma. Another clinical entity, in which the target of inflammation is thought to be the endothelium, has been recognized and recently classified as corneal endotheliitis.1,2

Clinical manifestation of corneal endotheliitis varies markedly. Since Khodadoust et al.3 reported a “presumed autoimmune corneal endotheliopathy,” in which they assumed that the disease resulted from an autoimmune reaction in the corneal endothelium, clinicians have noticed various forms of corneal endotheliitis. A variety of names have been given to this distinct clinical entity, including progressive herpetic corneal endotheliitis,4 herpetic endothelial keratitis,5 idiopathic corneal endotheliopathy,6 and sporadic diffuse corneal endotheliitis.7 Many patients with HSV disease may have corneal stromal edema that develops without stromal infiltrate. These patients all share the following findings: keratic precipitates (KPs), stromal and epithelial edema, and mild iritis. The KPs may be difficult to see because of extensive corneal edema but usually become evident as the stromal edema resolves. Many clinicians diagnose this clinical entity as stromal keratitis; however, it may more properly be considered to an inflammatory reaction of the endothelium, with only secondary stromal and epithelial edema.1,2

Although the exact pathogenesis of corneal endotheliitis is not known, accumulating evidence has indicated that viral origin is a strong possibility.1,2,4,8–15 The role of an infectious virus as the cause of endothelial infection is supported first by the detection of HSV-1 antigen, DNA, or viral particles in corneal endothelial cells.4,8–15 Second, endothelial lesions can rapidly resolve only with the addition of topical and systemic antiviral treatment.1,2,4,13–15

To date, most of the publications regarding corneal endotheliitis are clinical observations and case reports. To the best of our knowledge, there has been no report of an animal model of experimental corneal endotheliitis. The current experiments were undertaken to test our hypothesis that HSV-1 infection is one cause of corneal endotheliitis and that anterior chamber associated immune deviation (ACAID) is the underlying pathogenic mechanism for this entity. We established herpetic corneal endotheliitis in a rabbit model in the setting of induced ACAID.
MATERIALS AND METHODS

Animals

Normal Japanese albino rabbits (2.5–3.0 kg) were used. All animal procedures were performed using a combination of ketamine (70 mg/kg) and xylazine (10 mg/kg) as the anesthetic agent. In managing the rabbits we adhered strictly to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus

HSV-1 strain McKrae was used in these experiments. Viral stocks were propagated in primary rabbit kidney (PRK) cells grown in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated fetal bovine serum and antibiotics (Gibco, Grand Island, NY). An aliquot of passaged virus was titered by plaque assay on African green monkey kidney cell monolayers. Viral stocks were frozen in small amounts and stored at −80°C until use.

UV-Inactivated Virus

UV-inactivated HSV-1 (UV-HSV-1) was made by irradiating HSV-1 strain McKrae with UVB light at a distance of 12 cm for 16 minutes. The viral titer before irradiation was 2.5 × 10^7 plaque-forming units (PFU)/ml. After irradiation the virus showed no cytopathic effect on the PRK cells without compromised antigenicity, verified by immunocytochemical study.

Experiment 1: Herpetic Corneal Endothelitis Model

Experimental Design. Eighteen rabbits were randomly separated into three groups in equal number. One group received left-eye intracameral inoculation of UV-HSV-1 (3.8 × 10^6 PFU in 150 µl medium). The second group received PRK cell medium in the same manner as the first group. The third group received the same inoculum subcutaneously (SC) as the first group. Seven days later, the right eyes of all animals were intracameraly infected with 2.5 × 10^4 PFU of infectious HSV-1 contained in 100 µl medium.

After infection all rabbit eyes were monitored in a double-blind fashion by slit lamp examination every other day for 2 weeks. Blood samples were collected on postinfection (PI) days 0, 7, and 14. Samples from each group were pooled and detected for HSV-1-neutralizing antibodies.

At the conclusion of this experiment (PI day 14) rabbits were killed. The anterior segment of the eye including cornea, iris, and ciliary body was dissected and separated randomly into two parts. One part was examined with immunohistochemical staining. The other part of the cornea was examined with either scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Aqueous humor was also aspirated for detection of HSV-1 DNA and neutralizing antibody.

All experimental procedures were repeated three times, and HSV-1-neutralizing antibody and PCR samples were analyzed in triplicate.

Slit Lamp Examination. Rabbits were examined for the presence of inflammation in the anterior segment including corneal edema, KPs, and iritis. Each category was graded with a 0 (normal) to 4 (most severe) scoring system. Each clinical sign was evaluated by specific criteria. Corneal edema was scored as follows: 1, detectable thickening; 2, iris detail visible through edematous cornea; 3, iris detail not visible through edematous cornea; and 4, severe, whole cornea edema, peripheral iris not visible. KP was scored as follows: 1, detectable; 2, sporadic, small size; 3, diffuse, middle size; and 4, clumped, large size. Iritis was scored as follows: 1, sectorial redness; 2, redness of the whole iris, detectable flare cell in anterior chamber; 3, swelling and exudation of fiber protein, mild flare cell; and 4, frank hemorrhage and severe flare cell. The mean disease scores for corneal edema, KPs, or iritis were calculated for each group of rabbits on each slit lamp examination.

Detection of HSV-1 Antigen. Halves of the anterior segments of the eyes were fixed in periodate-lysine-paraformaldehyde fixative and immersed in a graded (10%, 20%, and 30%) sucrose series. After embedding in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA), specimens were snap frozen in liquid nitrogen and serially sectioned at 7-μm thickness by cryotome (Cryocut 3000; Leica, Wetlar, Germany). Sections were stained with monoclonal anti-HSV-1 antibody in a dilution of 1:100 (Quartett, Berlin, Germany). A peroxidase-standard kit (PK 4010; Vectastain; Vector, Burlingame, CA) was used, and the staining procedures followed the manufacturer’s instructions.

Electron Microscopy. For SEM, corneas were fixed in 3% glutaraldehyde, postfixed in 2% osmium tetroxide, mounted on stubs, sputter coated with gold-palladium, and observed (H-800; Hitachi, Tokyo, Japan). For TEM, specimens were fixed in 3% glutaraldehyde for 3 hours and postfixed in 2% glutaraldehyde for 2 hours. Specimens were dehydrated with a graded ethanol series and embedded in Epon 812. Ultrathin sections (70 nm) were cut, stained with 2% uranyl acetate and lead citrate solutions, and examined with TEM (H-800, Hitachi, Tokyo, Japan) at 100 kV.

PCR Analysis. Isogen reagent (Nippon Gene, Tokyo, Japan) was used to extract DNA from the aqueous humor samples according to the manufacturer’s instruction. DNA extracted from infected rabbit corneal cell suspension was used as the positive control. DNA extracted from normal rabbit cornea homogenate was used as the negative control.

Each PCR reaction contained 0.1 μg extracted DNA, 0.5 μM of primers specific for HSV-1 ribonucleotide reductase gene (sense: 5'-ATG CCA GAC CTG TTT TTC AA-3; and antisense: 5'-GTC TTT GAA CAT GAC GAA GG-3; this primer pair generates a 243-bp product), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 2.5 U of Taq polymerase (AmpliTaq; Perkin-Elmer, Foster City, CA) and 1× PCR buffer in a 50 μl final volume. Cycling reactions were performed in a thermal cycler (MJ Research, Waltham, MA). Each cycle included denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60 seconds. The final cycle was terminated with a 10-minute extension period at 72°C. Samples underwent 45 cycles of amplification. The amplified samples were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized using a digital imaging system (Eagle Eye; Stratagene, Seattle, WA).

HSV-1–Neutralizing Antibody Assay. Blood samples were centrifuged to harvest sera. Sera and aqueous humor samples were millipore sterilized with filters containing 0.2-μm pores (Corning, Westbury, NY). Microneutralization assays were performed according to the method described by Whittum et al.16


**Experiment 2: Verification of ACAID Induction after Intracameral Inoculation of UV-HSV-1**

Four groups of rabbits were treated as follows: group A, subconjunctival inoculation of $3.8 \times 10^6$ PFU UV-HSV-1 (150 μl); group B, no treatment; group C, intracameral inoculation of $3.8 \times 10^6$ PFU UV-HSV-1 (150 μl); and group D, intracameral inoculation of 150 μl PRK cell medium.

Seven days later, all groups except group D received an immunizing dose of UV-HSV-1 ($3.8 \times 10^6$ PFU) emulsified 1:1 in CFA (0.5 mg mycobacterium per milliliter, Gibco, Grand Island, NY). Two milliliters of this mixture was distributed SC in equal amounts at four body sites of each rabbit.

Seven days after immunization, delayed type hypersensitivity (DTH) was detected. The preinjected thickness of each rabbit ear (time zero) was measured using an engineer’s micrometer (Mitutoyo, Tokyo, Japan). DTH response was assayed by injecting 150 μl of $3.8 \times 10^6$ PFU erstwhile UV-HSV-1 into the ventral aspect of the right ear using a 250-μl Hamilton syringe (Wilmad, Reno, NV) equipped with a 30-gauge needle; the left ear served as the control. The amount of ear swelling was measured at 24 and 48 hours after challenge. Results are expressed as the amount of ear swelling at each time point by calculating the change in thickness of each ear from its respective time zero measurement and then subtracting the value of the control ear from that of the viral antigen-tested ear. All experimental procedures were performed three times.

**Statistical Analysis**

Statistical comparisons of the scores of the anterior segment inflammation between groups in experiment 1 and the comparisons of ear-swelling data between groups in experiment 2 were performed by conducting an initial one-way analysis of variance (ANOVA) on the inflammation score or the ear-swelling data, respectively. If the ANOVA results were significant ($P < 0.05$), comparisons were conducted using the overall ANOVA estimate of the relevant group mean error to conduct protected pair-wise t-tests. Comparisons of the frequency of HSV-1 DNA detection in aqueous humor samples were conducted by χ² test. Comparisons of the HSV-1-neutralizing antibody titers in blood and aqueous humor between groups were conducted by likelihood ratio test. $P < 0.05$ defines significance. All analyses were performed with computer software (Statistical Analysis System; SAS Institute, Cary, NC).

**RESULTS**

**Clinical Findings**

In experiment 1, rabbit eyes were evaluated by slit lamp examination for 14 days after right eye infection. Experiments were performed three times, each of which produced very similar results. In general, all left eyes showed no signs of evident inflammation and no difference between groups. Examination of right eyes revealed striking findings. A representative result of slit lamp examination on the right eyes is depicted in Figure 1.

As shown in Figures 1B and 1C, rabbits intracamerally inoculated with PRK cell medium or SC inoculated with UV-HSV-1 1 week before the anterior chamber infection developed intense, nonspecific inflammation in the anterior segments of the eyes. Viral infection was manifested by moderate to severe ciliary injection, KPs, diffused corneal edema and severe iritis. The inflammatory reaction peaked on PI days 6 to 8 and gradually subsided by PI day 14. On PI day 6, clinical scores were 2.5 ± 0.41, 2.25 ± 0.42, and 3.53 ± 0.26 for corneal edema, KPs, and iritis, respectively, in rabbits receiving left-eye intracameral inoculation of PRK cell medium 1 week before right-eye infection. Scores were 2.67 ± 0.44, 2.42 ± 0.38, and 3.08 ± 0.38 for corneal edema, KPs, and iritis, respectively, in rabbits receiving SC inoculation of UV-HSV-1 before infection.

There were no statistical differences in the severity of corneal edema ($P = 0.24$), KPs ($P = 0.22$), or iritis ($P = 0.1$) between these two groups.

In sharp contrast, rabbits that were intracameral inoculated with UV-HSV-1 1 week before infection showed profoundly different ocular inflammatory patterns (Fig. 1A). During the first week after infection, the infected eyes showed development of relatively mild inflammation in the anterior segments. Inflammatory reactions were mainly noticed on the cornea, which displayed edema to the same extent as the other two groups. A relatively “silent” anterior chamber with very little flare cell reaction and mild iritis was the sign of the ocular infection in this group. On PI day 6, clinical score was 0.43 ± 0.2 for iritis. Compared with the scores for the other two groups on the same observation day, the difference was significant ($P < 0.001$). During the second week of infection and by PI day 14, a distinct inflammatory pattern developed in the anterior segment that very much resembled the clinical features in some patients with corneal endotheliitis. The inflamed eye had a large, semicircular area of stromal edema overlying KPs in the lower part of the paracentral cornea. The ground-glass appearance of the corneal edema presented a distinct clinical picture with a definite focal pattern and definite demarcation between involved and uninvolved cornea. There was very mild inflammation in the anterior chamber (Fig. 2A). Several clumps of the characteristic KPs laid underneath the distribution of stromal edema, best seen by oblique illumination (Fig. 2B).

**Electron Microscopic Findings**

To better observe the details of the corneal endothelial layer, we performed electron microscopy studies. Corneas from rabbits that received intracameral inoculation of PRK cell medium or SC inoculation of UV-HSV-1 showed morphologically normal endothelia with very few visible defects. In marked contrast, corneas with herpetic endotheliitis appeared to possess many inflammatory cells in the endothelial layer. Enlarged intercellular gaps and infiltration of inflammatory cells characterized the numerous endothelial defects as shown by SEM (Fig. 3A). Interestingly, some spotty “holes” were distinguishable in the endothelial layer (Fig. 3B). TEM results showed that the infiltrating cells had the morphologic features of lymphocytes, plasma cells, or polymorphonuclear cells that incorporated into the destroyed endothelial layer of the endotheliitis-affected cornea (Fig. 3C).

**Viral Antigen Detection**

Anterior segments of the infected right eyes were investigated to determine the existence and distribution of HSV-1 antigen. In a typical experiment, for rabbits receiving intracameral inoculation of UV-HSV-1, viral antigen was detected only in the endothelial layer of all six samples. One exception was that, in
FIGURE 1. Slit lamp examination of inflammatory reactions in the eyes of three groups of rabbits. One group (A) received left-eye intracameral inoculation of UV-HSV-1 ($3.8 \times 10^6$ PFU). The second group (B) received PRK cell medium in the same manner as the first group. The third group (C) received the same inoculum as the first group, administered subcutaneously. Seven days later, the right eyes of all animals were intracamerally infected with $2.5 \times 10^4$ PFU of infectious HSV-1. Inflammatory reactions including corneal edema ($\triangle$), KPs (■), and iritis (○) were graded with a 0 (normal) to 4 (most severe) scoring system. Each data point represents the mean disease score of six rabbits per group.
addition to the endothelial layer, one eye also had viral antigen detectable in the anterior part of the ciliary body. In sharp contrast, all samples from rabbits that received intracameral inoculation of PRK cell medium (six samples) or SC inoculation of UV-HSV-1 (six samples) showed negative results in all parts of the anterior segments examined. A representative result of the HSV-1 antigen detection using immunohistochemical staining from a cornea with endotheliitis is shown in Figure 4.

Detection of HSV-1 DNA in Aqueous Humor

Aqueous humor aspirates harvested on PI day 14 were examined for HSV-1 DNA. Combining the data from three separate experiments, viral DNA was detected in 14 of 18 (77.8%) samples from rabbits that intracamerally received viral antigen 1 week before infection. Four of 18 (22.2%) samples from rabbits that intracamerally received PRK cell medium had detectable viral DNA. Only 2 of 18 (11.1%) samples from rabbits that received UV-HSV-1 SC were positive. A $\chi^2$ test verified that there were significant differences in the frequency of HSV-1 DNA detection between rabbits that received viral antigen intracamerally and the other two groups (with cell medium, $P = 0.003$ and with SC UV-HSV-1, $P = 0.001$). A representative result of the PCR analysis is shown in Figure 5.
HSV-1 Antibody Detection

Three separate experiments were performed, and in each experiment all samples were analyzed in triplicate. A representative result from one assay of peripheral blood is shown in Table 1. Results of the HSV-1–neutralizing antibody detection in aqueous humor samples (PI day 14) are shown in Table 2. Rabbits intracameraly inoculated with UV-HSV-1 were able to produce HSV-1 antibodies in peripheral blood at the time of anterior chamber infection (1:32), in comparison with less than 1:1 for rabbits intracameraly inoculated with PRK cell medium and 1:4 for rabbits primed SC with viral antigen (Table 1). A likelihood ratio test showed that the differences in HSV-1 antibody titer between rabbits intracameraly inoculated with viral antigen and the other two groups were significant ($P < 0.001$). Moreover, on PI day 14, the aqueous humor samples from rabbits in which corneal endotheliitis had developed showed a significantly higher antibody titer in comparison with the other two groups (Table 2, likelihood ratio test, $P < 0.001$). The HSV-1 antibody titer in aqueous humor samples from rabbits receiving PRK cell medium was not significantly different from that of rabbits receiving SC inoculation of viral antigen (likelihood ratio test, $P = 0.258$).

ACAI D Induction by Intracameral Inoculation of HSV-1 Antigen

To test our hypothesis that ACAID is the underlying mechanism of this herpetic corneal endotheliitis model, experiments were performed to verify that anterior chamber inoculation of UV-HSV-1 could result in an impairment of virus-specific DTH response and an intact humoral antibody-forming ability, which are hallmarks of ACAID.

Three experiments were performed, each of which produced data similar to that shown in Table 3. A summary of the three experiments is displayed in Figure 6. Our pilot experiments also included one group in which rabbits were treated with anterior chamber inoculation of PRK cell medium followed by immunization. This group was found to be no different from group B (no treatment). Rabbits that received intracameral inoculation of UV-HSV-1 (group C) showed significantly impaired DTH responses to HSV-1 antigen. In three experiments, the ear-swelling responses in group C were only approximately 40% as intense as the subconjunctivally inoculated, DTH-positive control animals (group A, $P = 0.006$).

The ear-swelling data of rabbits that received UV-HSV-1 intracameraly (group C) was not significantly different from the values obtained for rabbits that received PRK cell medium intracameraly (group D). The DTH response of rabbits receiving subconjunctival inoculation of UV-HSV-1 (group A) was not significantly different from that of rabbits receiving immunization alone (group B, $P = 0.247$).

Another important hallmark of ACAID is the ability to produce antibodies to the inoculated viral antigen, even though systemic DTH to this antigen is impaired (undisturbed antibody production). An HSV-1–neutralizing antibody assay in experiment 1 showed that rabbits intracameraly primed with HSV-1 antigen were able to produce HSV-1–neutralizing antibodies in peripheral blood (1:32, Table 1). More important, on PI day 14, rabbits with corneal endotheliitis had significantly higher titers of HSV-1 antibodies in the aqueous humor than did the other two groups (Table 2).

Thus, ACAID induction in rabbits after intracameral inoculation of UV-HSV-1 was confirmed. The pattern of impairment of DTH reactivity with concomitant production of neutralizing antibody to the virus is typical of ACAID.

Failure of Corneal Endotheliitis Induction by Systemic Immunosuppression

Because the specific immune deviation of ACAID is that the cell-mediated immune response is selectively impaired, whereas humoral immune responses are undisturbed or enhanced, we investigated whether systemic immunosuppression (both cell-mediated and humoral immune responses) could similarly promote herpetic corneal endotheliitis. Panels of rabbits were immunosuppressed with both 200 mg cyclophosphamide given intravenously on days 1, 4, 8, and 12 and 10 mg trimcinolone administered SC daily. The effect of immunosuppression was confirmed by white blood cell count in peripheral blood. Seven days later, rabbits were intracameraly inoculated with $2.5 \times 10^3$ PFU of infectious HSV-1 and monitored by slit lamp examination.

All infected eyes showed development of very severe viral endophthalmitis and stromal keratitis. No corneal endotheliitis was found. Neither peripheral blood nor aqueous humor samples showed an increase in the titer of HSV-1 antibody throughout this experiment (data not shown). Therefore, systemic

<table>
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<th>Group</th>
<th>Post Infection Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
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</tr>
<tr>
<td>AC-antigen</td>
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</tr>
<tr>
<td>AC-medium</td>
<td>1:4</td>
</tr>
<tr>
<td>SC-antigen</td>
<td>1:1</td>
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</table>

Eighteen rabbits were separated into three groups. One group (AC-antigen) received left-eye intracameral inoculation of UV-HSV-1 ($3.8 \times 10^8$ PFU in 150 μl). The second group (AC-medium) received PRK cell medium in the same manner as the first group. The third group (SC-antigen) subcutaneously received the same inoculum as the first group. Seven days later, the right eyes of all animals were intracameraly infected with $2.5 \times 10^3$ PFU of infectious HSV-1 contained in 100 μl. Peripheral blood samples were collected on PI days 0, 7, and 14. Samples from each group were pooled and examined for HSV-1–neutralizing antibodies by microneutralization assay. The naive group consisted of two untreated rabbits used for an antibody negative control. AC, anterior chamber.

*All experimental procedures were performed three times. In each experiment, blood samples were analyzed in triplicate. Data shown here are a representative result from one assay.

TABLE 1. HSV-1–Neutralizing Antibody Detection in Peripheral Blood
TABLE 2. HSV-1–Neutralizing Antibody Detection in Aqueous Humor at PI Day 14

<table>
<thead>
<tr>
<th>Experiment</th>
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<th>AC-medium</th>
<th>SC-antigen</th>
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<td>1:16</td>
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\( P^* \) <0.001

Eighteen rabbits were separated into three groups. One group (AC-antigen) received left-eye intracameral inoculation of UV-HSV-1 \( (3.8 \times 10^6 \text{ PFU in } 150 \mu l) \). The second group (AC-medium) received PRK cell medium in the same manner as the first group. The third group (SC-antigen) subcutaneously received the same inoculum as the first group. Seven days later, all right eyes were intracamerally infected with \( 2.5 \times 10^3 \text{ PFU of infectious HSV-1 contained in } 100 \mu l \). Aqueous humor aspirates were obtained from the right eyes on PI day 14. Samples from each group were pooled and examined for HSV-1–neutralizing antibodies by microneutralization assay. All experimental procedures were performed three times. In each experiment samples were analyzed in triplicate. The naive group consisted of two untreated rabbits used for an antibody negative control. AC, anterior chamber.

*Statistical comparisons of the titers of HSV-1–neutralizing antibodies between the AC-antigen group and the other groups were conducted by a likelihood ratio test. \( P < 0.05 \) defines significance.

**DISCUSSION**

Our interest and attention to conduct experiments testing a role for ACAID in herpetic corneal endotheliitis stems from the common clinical finding that mild inflammation of the anterior chamber accompanies corneal endotheliitis in patients. Why, if herpetic infection exists in these eyes, as suggested by a large number of reports,1,2,8–15 is the inflammation in the anterior chamber? We have reasoned that the answer may lie in a unique immune response that occurs when antigens enter the anterior chamber. ACAID, first described by Streilein and associates20–24 offers a potential explanation for this. DTH is a powerful inducer of immunogenic inflammation in which interferon-\( \gamma \) is a prominent effector cytokine. Because antigenspecific DTH is selectively suppressed in ACAID, we hypothe-

TABLE 3. DTH Responses in Rabbits

<table>
<thead>
<tr>
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<th>B</th>
<th>C</th>
<th>D</th>
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<td>293 ( \pm ) 26</td>
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<td>337 ( \pm ) 42</td>
<td>277 ( \pm ) 35</td>
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<tr>
<td>3</td>
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<td>765 ( \pm ) 80</td>
<td>512 ( \pm ) 33</td>
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<td>Total†</td>
<td>787 ( \pm ) 82</td>
<td>750 ( \pm ) 76</td>
<td>314 ( \pm ) 30‡</td>
<td>260 ( \pm ) 27‡</td>
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</table>

Four groups of rabbits were treated as follows: (A) subconjunctival inoculation of \( 3.8 \times 10^6 \text{ PFU UV-HSV-1} \), (B) no treatment, (C) intracameral inoculation of \( 3.8 \times 10^6 \text{ PFU UV-HSV-1} \), and (D) intracameral inoculation of PRK cell medium. Seven days later, all groups except group D received an immunizing dose of UV-HSV-1 emulsified 1:1 in complete Freund’s adjuvant. Seven days after immunization, DTH response was detected by injecting eerstwhile UV-HSV-1 into the ventral aspect of the rabbit ear to measure the ear swelling at 24 and 48 hours after challenge.

*Experiments were performed three times. Data are expressed as mean ear swelling (in micrometers) of five rabbits per group, in each experiment. 24 hours after ear challenge \( \pm \) SE.

†In three experiments combined, there were 15 rabbits in each group.

‡Mean swelling responses significantly less than positive control (group A).
sized that the existence of ACAID due to viral antigens may permit HSV-1 to invade and damage corneal endothelial cells, while avoiding the antiviral effects of T cells that mediate delayed type hypersensitivity.

Our data showed that rabbits primed with UV-HSV-1 through anterior chamber 1 week before infection developed a distinctive pattern of infection that very much resembled clinical corneal endotheliitis in patients. Immunohistochemical staining revealed that HSV-1 antigen was only detected in the destroyed endothelial cell layer. Therefore, the viral infection appeared to be limited to the corneal endothelium, implying that keratocytes, corneal epithelial cells, and iris and ciliary body tissues were protected from viral spread. Inflammatory cells including lymphocytes, plasma cells, and polymorphonuclear cells were found to infiltrate the region where endothelial cells were destroyed. The corneal edema that manifested in the infected eyes could reflect the destruction or dysfunction of the endothelial cell layer.

It is well known that HSV-1 can be transported in retrograde fashion to neuronal ganglia, such as the trigeminal ganglion, after primary infection of mucocutaneous tissues. Within the trigeminal ganglion, the virus usually establishes a long-term, latent infection that can become intermittently active. Low doses of virus may be spontaneously shed in the eye. It is likely that HSV-1 particles can be released into the anterior chamber through the trigeminal nerves that innervate the trabecular meshwork, the iris, and the ciliary body. Some of the viral particles can be captured by indigenous antigen-presenting cells, which, in turn, generate an immunogenic signal that induces virus-specific ACAID. We believe that the anterior chamber inoculation of UV-HSV-1 in our experiments produced ACAID by a similar mechanism. The reason we used viral antigen, not infectious virus, is that by injecting inactivated virus, a much higher concentration of viral antigen could be delivered without destruction of the anterior segment of the eye. This “silent” priming is a scenario very similar to that of clinical patients who have asymptomatic spontaneous viral shedding from low numbers of viral particles.

The amount of viral shedding during any particular reactivation may vary considerably. We suspect that when the dose of released virus is at or near 10^4 PFU, which is similarly produced in our rabbit model by intracranial infection of infectious HSV-1, the reactivating virus may travel into the immunosuppressive microenvironment of the anterior chamber and cause herpetic corneal endotheliitis. Actually, in our pilot experiments, we have attempted to infect with lower (10^3 PFU) or higher (10^6 PFU) doses of infectious HSV-1. But in neither case did we observe typical clinical manifestations of corneal endotheliitis. In our hands, only challenging the anterior chamber with 10^4 PFU of infectious HSV-1 produced this model, suggesting that only a specific dose of viral release into the ACAID-predominated eye can induce herpetic corneal endotheliitis. It is likely that there is a threshold amount of infectious virus that creates this disease and that the balance of ACAID and host effector responses plays an important role.

In ACAID, virus-specific DTH is suppressed and virus-specific cytotoxic T-cell responses are blunted but serum anti-HSV antibodies are present, or even enhanced. This spectrum of immune effectors makes it possible for HSV-1 to avoid lysis by class I-restricted T cells. We found that high titers of neutralizing antibodies were present in the eyes with corneal endotheliitis. We postulate that these antibodies effectively neutralized the extracellular virus, thus preventing the spread of virus into other cells and tissues within the anterior chamber. The rather minimal inflammation observed in the anterior segments of the eyes with endotheliitis supports our postulation.

There are two points to emphasize. First, the route of ocular priming is the key to the induction of herpetic corneal disease. Corneal endotheliitis did not develop in rabbits primed SC with viral antigen. Moreover, our pilot experiments showed that intrastromal inoculation of HSV-1 antigen followed by the same-eye intracamer al HSV-1 infection could promote stromal keratitis and keratouveitis, but not corneal endotheliitis. These findings suggest that the susceptibility of the cornea to the burden of keratouveitis or endotheliitis during HSV-1 infection is probably dictated by the unique features of the immune response elicited by antigens presented first to the immune system through the eye. Second, the corneal endotheliitis model results from conditions in which there is an antigen-specific suppression of cell-mediated immunity and intact humoral immune response (ACAI D). Nonspecific systemic immunosuppression, in which both cell-mediated and humoral immune responses are suppressed, cannot promote corneal endotheliitis. Theoretically, any ACAID-like immune response that leads to a pattern of impairment of cell-mediated immunity with concomitant production of neutralizing antibody to HSV-1 should be considered in the interpretation of the current data observed in our studies.

In summary, our experiments revealed that HSV-1 infection could be one cause of corneal endotheliitis. ACAID may play an important role in this distinct clinical entity. The strain of HSV-1, viral dose, and subtle changes in the immune status of the host may conspire to create the various features of corneal endotheliitis observed in clinics.

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References
10. Sundmacher R, Neumann–Haefelin D. Herpes simplex virus isolations from the aqueous humor of patients suffering from focal