Viral-Antibody Complexes in Canine Adenovirus Type I (CAV-1) Ocular Lesion: Leukocyte Chemotaxis and Enzyme Release

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Disciplines
Medicine and Health Sciences | Ophthalmology | Veterinary Medicine

Comments
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VIRAL-ANTIBODY COMPLEXES IN CANINE ADENOVIRUS TYPE 1 (CAV-1) OCULAR LESIONS: LEUKOCYTE CHEMOTAXIS AND ENZYME RELEASE

L. E. CARMICHAEL1, B. L. S. MEDICI1, S. I. BISTNER2, and G. D. AGUIRRE1
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Accepted October 24, 1974

ABSTRACT Canine adenovirus-type 1 (CAV-1)-antibody complexes caused severe anterior uveitis with corneal edema ("blue eye") when injected into the anterior chamber of normal dogs. The response of the anterior uvea to such immune complexes (IC) was similar to the spontaneously occurring disease. In the presence of complement (C'3), IC caused release of neutrophile chemotactic factors. Following phagocytosis of IC-C', leukocytes released lysosomal enzymes, as indicated by the presence of acid phosphatase in the surrounding medium. Membrane bound viral aggregates, presumably IC, were common in neutrophiles and in macrophages that had infiltrated the anterior chamber of opaque eyes that occurred after intravenous (IV) inoculation with attenuated CAV-1. These data were incorporated into a postulated scheme for the pathogenesis of CAV-1 uveitis with corneal edema.

KEY WORDS: DOG, CANINE ADENOVIRUS-1, ANTERIOR UVEITIS, CORNEAL EDEMA, IMMUNE COMPLEX, LEUKOCYTE CHEMOTAXIS, PATHOGENESIS.

(Cornell Vet., 1975, 65:331-351)
INFECTION by canine adenovirus type-1 (CAV-1) causes a
generalized, frequently fatal disease in young dogs; how­
ever the disease in older animals usually is mild, often being
recognized only by an increase in specific antibody or by the
appearance in recovered dogs of severe inflammation of the
anterior uvea and corneal edema (“blue eye”). Ocular mani­
festations also are seen following vaccination with attenuated
CAV-1 strains (4,9). Previous reports from this laboratory
(1,5,6) have described clinical and pathological features of the
ocular disease in relation to changes associated with viral
growth in the eye and the associated systemic and local im­
mune responses. It was hypothecated that the ocular lesions
were mainly a consequence of Type III (immune complex­
mediated) hypersensitivity, for a feature of the disease was
the demonstration of viral antigen and specific antibody in
the anterior uveal tracts of virtually all affected eyes. Also,
severe uveitis occurred after the intraocular injection of
canine adenoviral-antibody complexes (immune complexes).
Inflammatory responses were more severe if complement
(C') had been added to immune complex (IC) preparations
before injections were made. Extensive and severe panuveitis
also occurred within 4 hours following intravitreous inj ection
of non-living CAV-1 soluble antigens into eyes of immunized
dogs with high levels of complement-fixing and neutralizing
CAV-1 antibodies. In the latter instances, histological exam ­
ination of affected eyes indicated that recovery would not have
occurred.

In a recent study (1), ultrastructural changes were de­
scribed in the corneal endothelium of dogs at various times
during the course of experimental CAV-1 infection. During
the early stage of inapparent or mild uveitis, virus could be
isolated from the aqueous fluid and clusters of viral particles
were observed in the nuclei of occasional corneal endothelial
cells. Later, during the stage of severe anterior uveitis (usual­
ly 7 to 8 days after inoculation by the intravenous route),
virus was not isolated from the aqueous fluid, but viral anti­
gen and immunoglobulins could be demonstrated by immuno­
fluorescence within inflammatory cells which had infiltrated

CAV-1 ANTERIOR UVEITIS

MATERIALS AND METHODS

Immune complex preparations. Cornell-l strain CAV-1
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isolation, growth in dog kidney cell (DKC) cultures, and the
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followed by dialysis against pH 10.3 buffer (0.1 M glycine-
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gen, as evaluated by complement-fixation tests, was obtained.
Mixtures of viral antigen and varying amounts of anti-CAV-1
immunoglobulin solution, prepared by ammonium sulfate pre­
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time a floccular precipitate appeared in some tubes. Mixtures

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numbers of polymorphonuclear leukocytes, occurred as clus­
ters within a fibrin meshwork (keratic precipitates) and com­
monly were found closely adherent to the corneal endothelium.
Electron microscopic study revealed phagocytized aggregates
of membrane-bound CAV-1 particles within the cytoplasm of
apparently normal and degenerating macrophages and neu­
triphiles. Such aggregates were interpreted as IC within
phagolysosomes.

To further investigate the pathogenic functions of IC in
CAV-1 ocular disease, the generation of leukocytic chemotac­
tic factors (CF) and release of lysosomal hydrolases as pos­
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In this paper, additional evidence is presented that indicates
a pathogenetic role for viral-antibody complexes in the an­
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Infection by canine adenovirus type-1 (CAV-1) causes a generalized, frequently fatal disease in young dogs; however, the disease in older animals usually is mild, often being recognized only by an increase in specific antibody or by the appearance in recovered dogs of severe inflammation of the anterior uvea and corneal edema (“blue eye”). Ocular manifestations also are seen following vaccination with attenuated CAV-1 strains (4,9). Previous reports from this laboratory (1,5,6) have described clinical and pathological features of the ocular disease in relation to changes associated with viral growth in the eye and the associated systemic and local immune responses. It was hypothesized that the ocular lesions were mainly a consequence of Type III (immune complex-mediated) hypersensitivity, for a feature of the disease was the demonstration of viral antigen and specific antibody in the anterior uveal tracts of virtually all affected eyes. Also, severe uveitis occurred after the intraocular injection of canine adenoviral-antibody complexes (immune complexes). Inflammatory responses were more severe if complement (C') had been added to immune complex (IC) preparations before injections were made. Extensive and severe panuveitis also occurred within 4 hours following intravitreous injection of non-living CAV-1 soluble antigens into eyes of immunized dogs with high levels of complement-fixing and neutralizing CAV-1 antibodies. In the latter instances, histological examination of affected eyes indicated that recovery would not have occurred.

In a recent study (1), ultrastructural changes were described in the corneal endothelium of dogs at various times during the course of experimental CAV-1 infection. During the early stage of inapparent or mild uveitis, virus could be isolated from the aqueous fluid and clusters of viral particles were observed in the nuclei of occasional corneal endothelial cells. Later, during the stage of severe anterior uveitis (usually 7 to 8 days after inoculation by the intravenous route), virus was not isolated from the aqueous fluid, but viral antigen and immunoglobulins could be demonstrated by immunofluorescence within inflammatory cells which had infiltrated into the anterior chamber and trabecular spaces. The inflammatory cells, consisting of mononuclear and somewhat fewer numbers of polymorphonuclear leukocytes, occurred as clusters within a fibrin meshwork (keratic precipitates) and commonly were found closely adherent to the corneal endothelium. Electron microscopic study revealed phagocytized aggregates of membrane-bound CAV-1 particles within the cytoplasm of apparently normal and degenerating macrophages and neutrophiles. Such aggregates were interpreted as IC within phagolysosomes.

To further investigate the pathogenic functions of IC in CAV-1 ocular disease, the generation of leukocytic chemotactic factors (CF) and release of lysosomal hydrolases as possible mediators of corneal endothelial injury were examined. In this paper, additional evidence is presented that indicates a pathogenetic role for viral-antibody complexes in the anterior uvea of dogs infected with CAV-1. In addition to accounting for the sudden and extensive accumulations of inflammatory cells in the anterior chamber and trabecular spaces of affected eyes, in vivo and in vitro studies were made to attempt to relate lysosomal enzyme release to corneal endothelial damage.

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then were allowed to react at 4°C for an additional period of 24 hours, and the contents of the tube with the greatest amount of precipitate were combined with those of tubes with two- and four-fold greater amounts of antigen. The pooled precipitates then were centrifuged at 2,500 rev/min (PR-2 Centrifuge, International Eqpt. Co., Needham Heights, MA.) for 20 minutes at 4°C and washed twice in 0.15 M phosphate-buffered saline, pH 7.4 (PBS). After two additional washes, precipitates were suspended in PBS to one-fifth the original volume. Preparations of IC were stored at −15°C until used for dog inoculations or for in vitro experiments. Such IC preparations, after reaction with C′, consistently produced intense uveitis, commencing within 2–6 hours after injection into the anterior chamber of CAV-1 susceptible dogs’ eyes. Arthus-type skin reactions also occurred in dogs within 4–6 hours after intradermal inoculation of 0.2 ml amounts of IC into the abdominal skin.

After tests in dogs had demonstrated the capability of an IC preparation to engender anterior uveitis, the preparation was considered satisfactory for in vitro experiments.

Dog studies. A total of 66 specific-pathogen-free Beagle dogs from the Veterinary Virus Research Institute’s colony were used. Their ages ranged from 9 to 17 weeks. Inoculation of IC or lysed leukocyte suspensions into the anterior chamber of dogs’ eyes and collection of aqueous fluid samples was done as described elsewhere in detail (6). Selected eyes from dogs which had developed typical CAV-1 “blue eyes” following intravenous inoculation with attenuated CAV-1 were enucleated shortly after the onset of corneal opacification and stored in sealed plastic vials at −70°C until cryostat sections (4 nm) were made for immunofluorescent study. Eyes for electron microscopic examinations were fixed in situ, as described below.

Leukocyte preparations. Samples (16 ml) of peripheral blood were obtained from the jugular vein and placed in silicone-treated 50 ml glass centrifuge tubes containing 4 ml of sterilized 5% dextran (MW 250,000) made up in Hank’s saline solution, pH 7.4. The blood-dextran mixtures were allowed to stand for 1 hour at 35°C and the leukocyte-rich plasma portions, which always contained a small number of erythrocytes, were harvested and centrifuged for 5 min. at 800 rev/min. in an International PR-2 centrifuge. Cell pellets were then suspended to one-half the original plasma volume in RPMI 1640 medium containing 4% heated lamb serum. Leukocyte counts indicated 0.8 to 1.2 × 10⁷ cells/ml.

Electron microscopy. Ultrathin sections of portions of the anterior segment (cornea and angle) of eyes with early uveitis and corneal edema were fixed in situ and stained as described previously (1). For in vitro studies, leukocyte preparations were fixed with 2.5% glutaraldehyde and osmic acid and embedded for sectioning according to a described method (12). Examinations were done with a Philips EM-300 electron microscope.

Chemotactic factor (CF) assay. An assembly that contained two chambers separated by a membrane filter (AHLCO Mfg. Co., New Britain, Conn.) was used. Micropore filters (Millipore Corp., Bedford, Mass.), 0.65 nm pore size, were employed for the assay of CF elaborated by peripheral blood leukocytes. The method used has been described in detail (18). Immune complex–C′ (IC–C′) preparations to be tested for CF activity, together with appropriate controls, were injected into the lower compartment of a chemotaxis assembly; peripheral blood leukocyte suspensions were prepared as described above and 1 ml amounts then were injected into the upper chamber. After incubation for 3 hours at 37°C in 5% CO₂ and air, fluids were aspirated from the chambers and the filters were fixed and stained according to a described method (17). Quantitation of the leukotactic responses was done by counting (375 × magnification) the number of leukocytes that had migrated in at least 5 randomly selected fields. A chemotactic index (CI) was recorded (number of migrating cells reacted with test preparations divided by the number of leukocytes that had migrated when the lower chambers contained medium only). Only those cells which clearly had migrated into the plane of focus of the lower portion of the filter were counted. The 0.65 nm filter permitted evaluation only of neutrophile responses, since macrophages are excluded by the pore size of the filters used (18). Phago-
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Acid phosphatase assays. Acid phosphatase is a common marker enzyme, indicating liberation of lysosomal hydrolases from damaged leukocytes, or their release following phagocytosis of bacteria, zymosan particles, or immune complexes (20). In this study, acid phosphatase activity of aqueous fluid and serum samples from normal dogs and from dogs with CAV-1 induced corneal opacities were determined colorimetrically, using commercially prepared reagents (Sigma, St. Louis, Mo., Techn. Bull. No. 104, 1973). Similar tests were done using leukocyte suspensions (1 ml amounts) reacted in silicone-treated glass tubes with viral antigen, immunglobulin, or IC preparations (0.3 ml amounts), to which 0.1 ml of C3 had been added. In some instances, reactions were carried out on nonphagocytosable surfaces (Micropore filters, 0.45 nm pore size) according to described procedures (12,13). Enzyme activities were expressed as Sigma Units (Sigma, St. Louis, Mo., Techn. Bull. No. 104, 1973), or as per cent release of enzyme activity released into supernatant fluids obtained after centrifugation of leukocyte reaction mixtures for 10 min. at 2000 rev/min. at 4C. The total enzyme activity of leukocyte suspensions was liberated by treatment with 0.2% Triton X-100 (Rohm and Hass Co., Philadelphia, Pa.).

RESULTS

Neutrophile chemotaxis by IC. In the 6 dogs used for these experiments, uveitis commenced within 3 to 8 hours following intraocular inoculation with IC-C3, reaching maximal intensity approximately postinoculation (PI) hours 24-36 (Fig. 1). Reactions varied somewhat, but there was chemosis, iridal edema and marked aqueous flare in all injected eyes. Microscopic lesions of the anterior uveal tract shortly after onset of corneal edema consisted of heavy infiltrations by neutrophiles, with somewhat lesser numbers of mononuclear cells (Fig. 2). The cells appeared to originate from the root

![Fig. 1. Eye with acute anterior uveitis and corneal edema ("blue eye") observed 24 hours after intraocular injection with viral-antibody complex suspension. Keratic precipitates are adherent to the corneal endothelium.](image1)

![Fig. 2. Typical microscopic appearance of the trabecular meshwork of the irido-corneal angle as seen approximately 24 hours after the injection of immune complex-plus-complement suspension into the anterior chamber. Cellular infiltrate consists of neutrophiles (arrows) and monocytes. Azure II-methylene blue stain. X1400.](image2)
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of the iris, and were most numerous in the anterior chamber and trabecular spaces. The anterior chamber also contained large amounts of fibrin in which were enmeshed neutrophiles and monocytes. Areas of the corneal endothelium generally were absent, and the corneal-scleral junction was congested and infiltrated with large numbers of leukocytes. Viral isolations in DKC cultures were not made from ocular tissues or from aqueous fluid samples of dogs given IC-C'. Immunofluorescence studies revealed brightly staining aggregates of viral antigen in the cytoplasm of cells obtained from aqueous fluid smears (Fig. 3). Intranuclear virus was not observed.

Frozen sections of such eyes stained with fluorescein-labelled anti-dog C'3 revealed bright membrane-associated fluorescence of cells adherent to the corneal endothelium and within the trabecular spaces (Fig. 4).

Examination of frozen sections of eyes obtained from two dogs soon after the onset of corneal edema (PI days 7 and 8 following intravenous inoculation with attenuated CAV-1) also revealed viral antigen within cytoplasmic cellular compartments but, in contrast with dogs given intraocular injections with IC-C', intranuclear virus also was observed in small groups of corneal endothelial cells located approximately 1 to 3 mm from the corneal-iridal angle (Fig. 5).

Electron microscopic examination of eyes fixed shortly after the onset of corneal clouding revealed large numbers of cells (macrophages and neutrophiles) containing membrane-bound aggregates of adenoviral particles (Figs. 6a and 6b); some of the phagocytosed viral clusters appeared partially digested. Intranuclear (replicating) virus was not observed at this stage by electron microscopy. However, as noted above, immunofluorescence microscopy revealed occasional small clusters of cells, usually near the corneal-iridal angle, that contained intranuclear viral antigen. Attempts to isolate virus from such eyes were unsuccessful, probably because of
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neutralizing antibody which always is found in the aqueous fluid in opaque eyes (5).

![Image](image_url)

*Fig. 5.* Frozen section of the anterior segment of eye with corneal edema obtained 7 days after intravenous inoculation with CAV-1. Bright staining intranuclear viral antigen (arrow) in corneal endothelial cells. Aggregates of viral antigen also were seen in cytoplasm of clumped leukocytes within anterior chamber. Fluorescent antibody stain. X 400.

In *vitro* studies to determine the chemotactic activity of IC are summarized in Table 1. From these results, it was clear that chemotaxis by CAV-1/antibody complexes was dependent on the presence of fresh serum, i.e., a C' component(s). Figs. 7a, 7b and 7c illustrate the chemotactic responsiveness of peripheral blood leukocytes (neutrophiles) to IC-C'. Because results were essentially the same using IC preparations made from methanol-precipitated partially purified virus (Fig. 8) or viral subunit antigens prepared by dissociation at pH 10.3, data in Table 1 does not distinguish the tests done with these different antigens.

*Figs. 6a and b.* Double membrane-bound aggregates of viral particles within the cytoplasm of macrophage (M) juxtaposed against Descemet's membrane (D). Corneal endothelial cells were absent in this area (Fig. 6a). Electron photomicrograph, x25,000. Similar cytoplasmic structures (arrows) within macrophage comprising portion of keratic precipitate. Note fibrin (F). Electron photomicrograph, x13,000.
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Table 1. Neutrophile chemotaxis by canine adenovirus-1 (CAV-1)-antibody complexes (immune complexes)

<table>
<thead>
<tr>
<th>Test mixture*</th>
<th>Test no.</th>
<th>Mean number cells/field**</th>
<th>Chemotactic index (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC + fresh dog serum (C')</td>
<td>1</td>
<td>46</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt;150</td>
<td>&gt;15</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;100</td>
<td>&gt;21.7</td>
<td></td>
</tr>
<tr>
<td>IC + RPMI 1640 medium</td>
<td>1</td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>CAV-1 antigen + fresh dog serum (C')</td>
<td>1</td>
<td>5</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Anti-CAV-1 globulin + fresh dog serum (C')</td>
<td>1</td>
<td>3</td>
<td>1.0</td>
</tr>
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<td>2</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>RPMI 1640 medium</td>
<td>1</td>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>

*Test mixture indicates reactants in lower well of chemotaxis assembly, made up to final volumes of 1 ml with RPMI 1640 medium. Upper wells contained about 10^7 leukocytes, as described in text.

**At least 5 fields (40x objective magnification) counted per stained filter.

1IC = immune complex.

Figs. 7a, b and c. Chemotactic response of canine peripheral leukocytes (neutrophiles) to CAV-1-antibody complexes, plus complement (C'). Neutrophiles on top of filter membrane (7a) have migrated through filter pores to lower side (7b) in response to immune complex-C'-generated chemotactic factors. Absence of chemotactic factors (control) indicated by reduced leukocytic migration (7c). Arrow indicates single migrating cell within filter pore. Focus on top of filter, giving distorted image of cells on bottom of filter. H & E. X800.
Table 1. Neutrophile chemotaxis by canine adenovirus-1 (CAV-1)-antibody complexes (immune complexes)

<table>
<thead>
<tr>
<th>Test mixture*</th>
<th>Test no.</th>
<th>Mean number of cells/field**</th>
<th>Chemotactic index (Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC + fresh dog serum (C')</td>
<td>1</td>
<td>46</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>54</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;150</td>
<td>&gt;15</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;100</td>
<td>&gt;21.7</td>
</tr>
<tr>
<td>IC + RPMI 1640 medium</td>
<td>1</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>1.4</td>
</tr>
<tr>
<td>CAV-1 antigen + fresh dog serum (C')</td>
<td>1</td>
<td>5</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>1.4</td>
</tr>
<tr>
<td>Anti-CAV-1 globulin + fresh dog serum (C')</td>
<td>1</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>RPMI 1640 medium</td>
<td>1</td>
<td>***</td>
<td>***</td>
</tr>
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<td></td>
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**Lysosomal enzyme studies.** There was no measurable acid phosphatase activity in aqueous fluid samples from normal eyes, however elevated enzyme levels (0.1 to 1.0 Sigma units) were found in virtually all samples from opaque eyes (Table 2). Enzyme levels of serum samples obtained from 14 normal pig.

Immune complex preparation made from partially purified CAV-1 and antibody in region of “antigen excess.” Phosphotungstic acid stain. Electron photomicrograph. $\times$108,700.

Table 2. Acid phosphatase levels in aqueous fluid samples from normal eyes and eyes with corneal edema.

<table>
<thead>
<tr>
<th>Aqueous fluid samples</th>
<th>Acid phosphatase activity (Sigma units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal eyes (non-infected)</td>
<td>$&lt;.01$</td>
</tr>
<tr>
<td>Normal eyes (CAV-1 infected)**</td>
<td>18/18</td>
</tr>
<tr>
<td>Corneal edema (CAV-1 infected)+</td>
<td>30/39</td>
</tr>
</tbody>
</table>

*The numerator is the number of samples with acid phosphatase activity within the range listed; the denominator is the total number of samples tested in each class.

**Of the 39 samples in this category, 26 were from the non-affected eye of dogs with unilateral corneal opacities. The remainder were obtained from normal eyes of dogs at the time of onset of anterior uveitis with corneal edema in one or more littermates.

+Samples obtained within 12 hours after onset of corneal edema.

Table 3. Acid phosphatase release from canine leukocytes by canine adenovirus-1 (CAV-1)-antibody complexes (immune complexes).

<table>
<thead>
<tr>
<th>Test mixtures</th>
<th>Experiment</th>
<th>Acid-phosphatase (Units)$^*$</th>
<th>Per cent enzyme release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes + IC-C'**</td>
<td>1</td>
<td>3.6</td>
<td>6.9</td>
</tr>
<tr>
<td>Leukocytes + C' or immunoglobulin</td>
<td>2</td>
<td>6.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Leukocytes + 0.2% Triton X-100</td>
<td>3</td>
<td>6.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Leukocytes + C' or immunoglobulin</td>
<td>1</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Leukocytes + 0.2% Triton X-100</td>
<td>2</td>
<td>0.6</td>
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</tr>
<tr>
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<td>1.0</td>
<td>1.3</td>
</tr>
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</table>

$^*$Enzyme activity expressed as Sigma Units $\times$ 100. Values represent mean of at least three replicate tests.

**IC-C' indicates immune complex suspensions reacted with complement prior to addition of leukocytes.

The enzyme release from leukocytes reacted with IC-C' was $7.7 \pm 0.75\%$; in contrast, release from leukocytes incubated with CAV-1 antigen, C', or antiseraum amounted to only $1.1 \pm 0.01\%$. Reaction mixtures were monitored by immunofluorescence and, occasionally, by electron microscopy for phagocytic capabilities of the cell preparations. Active phagocytosis was revealed by the appearance of bright green cytoplasmic fluorescence in leukocytes reacted with IC-C' (Fig. 9). Also, cells that had ingested IC-C' occurred in clumps. Cells exposed only to viral antigen suspensions could not be distinguished from controls.

Electron microscopy of leukocytes exposed to IC-C' revealed heavily vacuolated cells, with some double membranous vacuoles containing clumps of viral particles and amorphous material. However, in the *in vitro* preparations,
Lysosomal enzyme studies. There was no measurable acid phosphatase activity in aqueous fluid samples from normal eyes, however elevated enzyme levels (0.1 to 1.0 Sigma units) were found in virtually all samples from opaque eyes (Table 2). Enzyme levels of serum samples obtained from 14 normal

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<tr>
<td>Leukocytes + IC-C'**</td>
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<td>6.9</td>
</tr>
<tr>
<td></td>
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<td>6.2</td>
<td>8.3</td>
</tr>
<tr>
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<td>1</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Leukocytes + 0.2% Triton X-100</td>
<td>1</td>
<td>82 ± 5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>82 ± 2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

**The numerator is the number of samples with acid phosphatase activity within the range listed; the denominator is the total number of samples tested in each class.

**IC-C' indicates immune complex suspensions reacted with complement prior to addition of leukocytes.

The enzyme release from leukocytes by IC-C' was 0.19 ± 0.09 Sigma units (range 0.07 to 0.41). Similar serum enzyme levels also were found in dogs with corneal edema. In 15 of 29 samples obtained from opaque eyes, enzyme levels of aqueous fluid samples were not significantly different from those of the serum; however 14 samples had acid phosphatase activities greater than found in serum, suggesting some local release.

**Table 2. Acid phosphatase levels in aqueous fluid samples from normal eyes and eyes with corneal edema.**

<table>
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<tr>
<th>Aqueous fluid samples</th>
<th>Acid phosphatase activity (Sigma units)</th>
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<th></th>
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</thead>
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<tr>
<td>Normal eyes (non-infected)</td>
<td>&lt;.01</td>
<td>0.1-0.3</td>
<td>0.4-1.0</td>
</tr>
<tr>
<td>Normal eyes (CAV-1 infected)**</td>
<td>18/18</td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>Corneal edema (CAV-1 infected)†</td>
<td>39/39</td>
<td>0/29</td>
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phagocytosed IC never were as compact as those observed in leukocytes within the anterior chamber of dogs with acute anterior uveitis and corneal edema.

Fig. 9. Canine peripheral leukocytes reacted with CAV-1-antibody complexes, plus complement. Cells are clumped. Bright areas represent cytoplasmic fluorescence of phagocytized CAV-1 antigen. Fluorescent antibody stain. X250.

DISCUSSION

When the present studies were initiated, it had been established that two distinct types of ocular reactions may occur in the dog following infection with CAV-1: (I) An intense uveitis with corneal edema and systemic signs occurs within 48 to 72 hours following injection of virus directly into the anterior chamber (10); (II) A delayed reaction may occur 1 to 3 weeks following the natural disease or after vaccination with attenuated virus consisting of mild to severe anterior uveitis, often with corneal edema—"blue eye"—(4,5,9).

The pathogenesis of the first, unnatural, condition is readily understood. Viral growth occurs rapidly in cells of the corneal endothelium and trabecular meshwork with extension of the inflammation to the posterior uvea. As a result, there is total loss of the corneal endothelium, cataractous changes in the lens, and severe uveitis that invariably terminates in total loss of vision in the inoculated eye. The second, spontaneous, type of ocular response to CAV-1 is more complex, for anterior uveitis without corneal edema is more common following CAV-1 infection (or vaccination) than "blue eye" (4).

In a recent ultrastructural study (1) of the changes in corneal endothelium following CAV-1 infection intranuclear viral particles were observed in corneal endothelial cells during the stage of mild anterior uveitis, prior to onset of corneal edema, augmenting previous virological studies (5). Later, at the time of onset of severe anterior segment inflammation with corneal edema, there was sequential infiltration of the anterior chamber and trabecular spaces, initially by neutrophils and, some hours later, by macrophages. These cells were found to contain viral aggregates within phagolysosomes in the cytoplasm. The aggregates were regarded as viral-antibody complexes.

In the present series of experiments, observations were extended to account for the very rapid onset of the corneal lesions and the keratic precipitate formation. The role of IC and complement in experimental tissue injury has been reviewed by several authors (7,8,19). As regards the eye, naturally occurring immunologically mediated disease is less clearly documented; however, convincing evidence has been compiled (2,3) for a pathological role for viral-antibody complexes in diseases of other organs, especially the kidney and vascular system. A notable difference between Type III reactions as described for experimental immunologic uveitis (15) and ocular lesions of CAV-1 is the localization of virus principally within the avascular corneal endothelium and trabecular meshwork cells, rather than within arterioles of the uveal tract. It was shown previously (1,5) that the presence of occasional viral-infected cells in the corneal endothelium does not generally lead to sufficient functional change to cause osmotic flow of aqueous fluid into the corneal stroma and result in edema. Rather, other mechanisms also must operate. The data presented here have demonstrated the
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in vitro release of a neutrophilic chemotactic factor by virus-antibody complexes plus C'. Also, the finding of cellbound C' by immunofluorescence in lesions, along with viral-antibody aggregates, provided further evidence for the Type III hypersensitivity reaction. The actual cause of functional corneal endothelial damage was not established by these studies; however, the finding, in some instances, of elevations in aqueous fluid acid phosphatase levels, sometimes above serum enzyme levels, suggests release, and a possible role, of lysosomal enzymes in tissue damage. Neutrophilic lysosomal extracts have been reported to cause reversible changes in cultures of corneal epithelial cells (14), however in recent experiments done in our laboratory, we were unable to observe severe damage to cultured corneal endothelial cells using similar methods. The failure to cause corneal endothelial damage may have been due to the rapid washing out of the small molecular weight acid proteases, since the trabeculae were not occluded by infiltrated cells, as occurs in spontaneous CAV-1 eye lesions. Also, adherence of large numbers of leukocytes and fibrin to the corneal endothelium as keratic precipitates could focus a sustained release of injurious constituents such as acid proteases, collagenase, and permeability factors (7,11,13). Such a mechanism is likely, since lesions are relatively focal and corneal edema generally resolves within a few days, sometimes weeks. The clumping of neutrophiles and monocytes within the plasmoid aqueous fluid may represent an immune adherence phenomenon, since in vitro observations indicated clumping of cells only in the presence of C'. Removal of the stimulus (IC-leukocytes) then allows repair of tissue damage if corneal endothelial destruction were not extensive.

In vitro release of acid phosphatase from leukocyte suspensions by IC-C' amounted to approximately 7.7%, as compared with the total enzyme activity released by a detergent (Triton X-100). The quantitative significance could not be assessed; however, similar release of acid phosphatase from human neutrophiles exposed to immune complexes has been reported (20,21) as indicative of liberation of lysosomal hydrolases during phagocytosis.

Based on these data, a postulated scheme for the pathogenesis of "blue eye" is presented (Fig. 10).

VIREMIA: Viral growth in RE tissue, kidney, liver
+ VIRAL GROWTH IN ANTERIOR UVEA/CORNEAL ENDOTHELIUM (CE)
  No growth -- no uveitis
  Growth -- CE, vascular endothelium, trabeculae

LOCAL IMMUNE RESPONSE
  Lymphocytes and plasma cells in anterior uvea
  Restricted viral growth

VIRAL GROWTH ARRESTED OR VIRAL PERSISTENCE IN RESTRICTED SITES
  No persistence -- no clinical signs
  Persistence -- CE cells, trabecular meshwork cells

IMMUNE COMPLEX FORMATION (CAV-1/antibody)
  Virus/antibody excess: slight or no pathogenicity
  Virus/antibody balance: complement (C') binding; pathogenic

C'-MEDIATED RELEASE OF BIOLOGICALLY ACTIVE COMPONENTS
  Leukocyte chemotactic factors
  Immune adherence factor
  Enhanced phagocytosis of immune complexes

LEUKOCYTE INFILTRATION INTO ANTERIOR CHAMBER

PHAGOCYTOSIS OF IMMUNE COMPLEXES-C' BY NEUTROPHILES AND MACROPHAGES

LYSOSOMAL ENZYME RELEASE
  Injury (focal) to CE at points of contact with leukocytes?

INFILTRATION OF LEUKOCYTES BETWEEN CE AND DESCemet's MEMBRANE
  Aqueous fluid permeates into corneal stroma
  Corneal edema

DIGESTION OF IMMUNE COMPLEXES WITHIN PHAGOCYTIC VACUOLES & REMOVAL
  If lesions in CE focal: resolution in 1-3 weeks
  If lesions extensive: no CE regeneration, persistent or permanent corneal damage. Bullous keratopathy, hydrophthalmos, or extensive corneal pigmentation with complete loss of vision.

Fig. 10. Postulated scheme of pathogenesis of acute anterior uveitis following CAV-1 infection. The pathogenetic role of lysosomal enzyme release from leukocytes is not fully established.
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Leukocyte infiltration into anterior chamber
Phagocytosis of immune complexes—C' by neutrophiles and macrophages

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