In Situ Immune Complex Formation with the Uvea

Potential Role of Cationic Antibody

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A murine model has been developed to study the role of immunoglobulin charge in the regulation of the intraocular distribution of circulating IgG antibodies. Intravenously injected cationic antibodies to the tracer enzyme horseradish peroxidase bind within the ciliary body and choroid (CB/Ch). These cationic antibodies can selectively entrap and bind circulating antigens forming immune complexes (IC) within the uveal tissues. The structure of the uvea with its fenestrated CB/Ch capillaries and fixed anionic sites (within Bruch’s membrane and the stroma of the CB and processes) may predispose the CB/Ch to in situ IC formation. Local IC formation mediated initially by deposition of cationic antibodies within the uvea may play an important role in the immunopathogenesis of some forms of uveitis.

Materials and Methods. Chemical modification of anti-HRP: Cationic rabbit anti-HRP (ca-a-HRP) (IgG fraction, Sigma Chemical Co., St. Louis, MO) is prepared as described by dissolving 10–20 mg antibody (native a-HRP) in 2.5 ml 0.01 M NaCl at 25°C. The solution is added to 4.4 ml 2 M ethylenediamine and adjusted to pH 7.0 with 2.0 N HCl. While stirring, 400 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, HCl is dissolved; the pH is maintained at 7.0 with 0.2 N HCl for 2 hr. The reaction mixture is kept overnight, concentrated by ultrafiltration, and dialedyzed against normal saline. The charge of cationic anti-HRP determined by isoelectric focusing is in the range of pI 9.0–10.0.

Detection of tissue-bound ca-a-HRP antibodies: The experimental protocol for the detection of tissue-bound ca-a-HRP has been modified from procedures used to demonstrate specific antibody-forming plasma cells within frozen lymphoid tissue sections. Briefly, 200 μg ca-a-HRP is injected into the lateral tail veins of experimental groups containing 20–25 6-week-old BALB/c mice (see Results for experimental details). After 30 min (and up to 5 days later) the animals are sacrificed by methoxyflurane inhalation and frozen sections prepared from the whole globe, spleen, kidney, and lymph node (non-ocular control tissues). Sections (4–6 μ) are placed onto chrome-alum coated slides, fixed in cold acetone and incubated in H2O2/methanol for 10 min to inactivate endogenous peroxidase activity. After washing in tris-saline, the sections are blocked with 3% BSA, 0.25% gelatin, 5 mM EDTA, and 0.05% NP-40, and incu-
bated with 50 μg/ml HRP (Sigma, Type II) in tris-saline for 20 min. Washed sections are developed with aminoethylcarbazole (AEC). Visual red precipitate within the tissue is indicative of tissue-bound anti-HRP antibodies.

Formation of in situ immune complexes within the uveal tract: A modification of the procedure of glomerular in situ IC formation, described by Agodoa et al., has been used to study parameters regulating local in situ IC deposition within the eye. Experimental groups of 20 Balb/c mice were passively immunized IV with 200 μg of caa-HRP antibodies. Twenty-four hr after binding of caa-HRP antibodies within the ocular tissues, 2 mg of HRP antigen was given IV. After an additional 24 hr, the mice were sacrificed and eyes were stained for the presence of antibody, complement, antigen, and inflammatory cells. Antibody (rabbit-IgG) was detected within frozen tissue sections by the biotin-avidin ABC (Vector Laboratories, Burlingame, CA) immunoperoxidase technique. Complement (C3) was detected within frozen sections using peroxidase-conjugated goat anti-C3 (IgG fraction, Cooper Biomedical, Malvern, PA). Antigen (HRP) was detected by direct AEC staining of 1-μ plastic sections (JB4, Polysciences, Inc., Warrington, PA) without H2O2/MeOH inactivation, blocking steps, and additional HRP incubation. Inflammatory cells were identified in 1-μ plastic sections stained with hematoxylin and eosin.

Additional contrasting experiments were carried out to determine the intraocular distribution of preformed IC following IV injection. IC were prepared in vitro with monomeric native anti-HRP and HRP in five-fold antigen excess. After centrifugation for 20 min at 1000 g, IC containing 200 μg of anti-HRP antibody was injected IV. At varying times, two or more animals were sacrificed, their eyes fixed in neutral buffered formalin, embedded in JB4, and stained for IC by incubation with AEC and counter-stained with Mayer’s Hematoxylin. Control animals received intravenously either 200 μg native anti-HRP antibodies or 2 mg HRP and were monitored as described for the intraocular distribution of antibody, antigen, and/or the non-specific induction of intraocular inflammation. All procedures used in this study conform to the ARVO Resolution on the Use of Animals in Research.

Results. IV injected caa-HRP (pI 9.0–10.0) is rapidly cleared from the circulation, being selectively deposited in 100% of animals (25/25) (at 30 min) within localized areas of the eye, spleen, and kidney. In the spleen, caa-HRP antibodies were bound in the sinu-
Inflammatory cells in the anterior chamber following in situ immune complex formation. Twenty-four hr after injection of cationic anti-HRP, mice were given intravenous HRP and sacrificed 24 hr later (Hematoxylin and Eosin, X250).

soids. The kidney glomeruli were also stained, indicating deposited caa-HRP. In ocular tissues, caa-HRP was deposited in the stroma of the CB and ciliary processes (CP) and not extending into the double-layered ciliary epithelium (Fig. 1). In the Ch, staining was observed throughout Bruch’s membrane and in the vessel walls of the choriocapillaris and stromal tissues (Fig. 1). Little or no staining was observed in the deeper Ch vessel walls. No staining was observed in the cornea, iris, lens, or retina. In (100%, 5/5) control animals injected with equivalent concentrations of native α-HRP (pi 5.5-7.7), faint staining was observed at 30 min in the splenic sinusoids and kidney glomeruli, but not in any ocular tissue. These experiments suggest that immunoglobulin charge is an important factor in the regulation of intraocular distribution of circulating antibodies (submitted for publication). Although caa-HRP staining was intense within the glomeruli, within 24 hr no caa-HRP was detectable. In contrast to the kidney, the CB/Ch-bound caa-HRP antibodies were detectable for up to 5 days in 4/5 animals following IV inoculation (the latest time point examined). These results suggest that the antibody tissue half-life or clearance mechanisms are different between the eye and the kidney.

The previous experiments demonstrate that caa-HRP antibodies can be actively and selectively localized from the circulation within specific areas of the uveal tract. The ability of this localized caa-HRP to induce in situ uveal IC was subsequently determined following IV injection of HRP.

In preliminary experiments (data not shown) using HRP alone as a tracer molecule, it was determined that IV HRP results in a transient deposition of HRP antigen within the CB/Ch of naive recipients, being rapidly cleared within 8–10 hr. In contrast, 18/20 mice given caa-HRP followed by HRP IV 24 hrs later, developed evidence of local uveal in situ IC within the CB/Ch (Fig. 1) lasting 24–48 hr. Within the CB/Ch, the distribution of HRP antigen was identical to that of rabbit IgG (data not shown). No antigen or antibody was detected in the cornea, lens, iris, sclera, or conjunctiva.

In contrast to the pattern of in situ-formed IC, the intraocular distribution of circulating preformed IC is much different. Preformed IC preferentially localized in 5/5 animals within the limbal-scleral capillary area (Fig. 1). Occasional, scattered deposits were also detected within the iris and choriocapillaris. Preformed IC were cleared from the eye within hours.

Although minor evidence of intraocular inflammation was detectable in most animals following in situ IC formation, only 10% (2/20) demonstrated a significant influx of polymorphonuclear leukocytes at 24 hr following IV-HRP (Fig. 2). Preformed IC did not induce any evidence of intraocular inflammation. Cationic antibodies bound within uveal tissues can thus selectively entrap and retain transient, circulating antigens and lead to in situ IC formation, and under certain circumstances induce inflammation within the CB/Ch.

Discussion. The anatomical structure of the uveal tract (CB/Ch) may predispose this ocular tissue to in situ IC formation leading to uveitis. The combination of fenestrated capillaries in the CB/C and anionic sites within the CB, ciliary processes and Bruch’s membrane provides a system which (like the glomerulus) is selectively permeable, retaining molecules of a given size and charge. The previous experiments demonstrate that caa-HRP antibodies can be actively and selectively localized from the circulation within specific areas of the uveal tract. The ability of this localized caa-HRP to induce in situ uveal IC was subsequently determined following IV injection of HRP. In preliminary experiments (data not shown) using HRP alone as a tracer molecule, it was determined that IV HRP results in a transient deposition of HRP antigen within the CB/Ch of naive recipients, being rapidly cleared within 8–10 hr. In contrast, 18/20 mice given caa-HRP followed by HRP IV 24 hrs later, developed evidence of local uveal in situ IC within the CB/Ch (Fig. 1) lasting 24–48 hr. Within the CB/Ch, the distribution of HRP antigen was identical to that of rabbit IgG (data not shown). No antigen or antibody was detected in the cornea, lens, iris, sclera, or conjunctiva.

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Hronstrat that HRP, and similar molecules, freely penetrate through the CB/Ch fenestrations. HRP, however, is not tightly bound within the CB/Ch presumably due to its size and charge. In contrast, cationic probes such as cationic ferritin penetrate and are tightly bound by anionic sites in the CB and Bruch’s membrane. Similar findings have been reported in the kidney, where the fenestrated capillaries and anionic sites have been implicated as contributing factors in glomerulonephritis.

We have defined a system to analyze these factors and their importance in the immunopathogenesis of uveitis. Our results indicate that cationically charged antibodies (pI 9.0–10.0) can be bound for at least 5 days within the anionic sites of the CB/Ch. The intraocular distribution of antibody is regulated, at least in part, by its isoelectric pH (submitted for publication). Furthermore, these cationic antibodies bound within the CB/Ch can selectively entrap and bind circulating antigens which gain access to the CB/Ch tissues, leading to in situ IC formation and, in some instances, inflammation within the uvea. The intraocular distribution of preformed IC, following IV injection, is much different from that demonstrated with local in situ IC in our model. The preformed IC do not deposit within the CB/Ch; instead, they are only transiently demonstrated in the limbal-scleral capillary plexus (Table 1).

Using our in situ system, IC were demonstrated in the CB/Ch with only occasional evidence of inflammatory cells in the CB/Ch and anterior chamber at 24 hr. The observed inflammation was not due to endotoxin contamination since intraocular inflammation was not induced in control animals. Furthermore, experiments designed to elicit endotoxin-induced uveitis in mice have proven unsuccessful (J. C. Waldrep, unpublished data, and J. T. Rosenbaum, personal communication).

The absence of inflammation in 18/20 animals could be due to the time period studied or the IC lattice size formed in situ, which can be important in complement activation. Comparison of the electrophoretic and precipitating properties of the caa-HRP preparations used in these experiments revealed that although highly positively-charged, they had reduced precipitating activity. In addition, chemical modification of the Fc portion of IgG (such as cationization) has been demonstrated to alter its complement-fixing activity.

Although our system does not consistently lead to severe intraocular inflammation, it does establish the potential pathogenic role of cationic antibody in local IC formation within the uveal tract. Immunoperoxidase experiments designed to determine the role of complement fixation following in situ IC formation revealed considerable C3 in the CB/Ch of both experimental and control eyes. This suggests that complement (at least C3) is normally present in these ocular tissues, presumably by diffusion from the circulation. A similar observation has been made in the cornea.

Further experiments are in progress to refine this experimental system for study of the initial inflammatory events in uveitis mediated by cationic anti-

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Table 1. Tissue deposition and persistence of antibody and immune complexes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CB/CP</th>
<th>Ch</th>
<th>Limbus</th>
<th>Iris</th>
<th>Spleen</th>
<th>Kidney</th>
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<tbody>
<tr>
<td>caa-HRP*</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>30 min</td>
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<td>Native α-HRP†</td>
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<td>In situ IC‡</td>
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<td>48 hr</td>
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</table>

* 200 μg IV followed by analysis of frozen sections at 30 min, 24 hr, and 120 hr.
† 200 μg IV followed by analysis of frozen sections at 30 min.
‡ 200 μg caa-HRP antibody IV followed by 2 mg HRP antigen 24 hr later.

Eyes were assayed for in situ IC 24 and 48 hr later.

‡ Containing 200 μg caa-HRP IV followed by analysis of tissue sections at 30 min, 24 hr, and 48 hr.

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bodies. Since it is known that there is an inverse relationship between antigen charge and charge of the induced antibody, one might postulate that anionic antigens actively induce cationic antibody formation in vivo.

These natural cationic antibodies may then bind within the uvea, as demonstrated experimentally. Human IgG₁, with a reported pl of 6.5–9.5, and IgG₃ with a pl of 8.2–9.0, would have this capability. Subsequent vascular dissemination of the anionic antigen might then induce the in situ IC formation ultimately leading to uveitis.

**Key words:** cationic antibodies, immune complexes, horseradish peroxidase, uveitis, uveal tract

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**References**


**Immunopathology of Acute Experimental Histoplasmic Choroiditis in the Primate**

Anthony Anderson,* Clive Taylor,† Stanley Azen,‡ James V. Jester,§ Cheryl Hagerty,‡ L. David Ormerod,|| and Ronald E. Smith§*

The immunopathologic features of experimental acute histoplasmonic choroiditis were studied in the nonhuman primate. Using an indirect immunoperoxidase technique, a panel of hybridoma-derived anti-human monoclonal antibodies, recognizing distinct lymphoid cell and macrophage surface antigens, have been adapted for use in the primate system. Twenty-two individual foci of histoplasmonic choroiditis from five eyes were studied at time periods from 20 to 60 days post intracarotid injection of yeast phase *Histoplasma capsulatum*. A mononuclear and granulocytic cell infiltration was seen in all lesions. The predominant cell type was the CAPPEL+ T lymphocyte (suppressor/cytotoxic subset). Other cell types found in smaller numbers were OKT4+ T cells (helper/inducer subset), OK7+ (peripheral B lymphocytes), IgD+ (mantle B cells) and OKM1+ cells (macrophages and polymorphonuclear leukocytes). Herein, we present immunopathologic data on the acute phase of experimental ocular histoplasmosis. Invest Ophthalmol Vis Sci 28:1195–1199, 1987

The importance of inflammation in the late disciform macular disease of the ocular histoplasmosis syndrome (OHS) remains speculative. While subreti-