Antigen from the Anterior Chamber of the Eye Travels in a Soluble Form to Secondary Lymphoid Organs via Lymphatic and Vascular Routes

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PURPOSE. To determine the afferent pathways linking the anterior chamber (AC) of the eye to the secondary lymphoid organs.

METHODS. Single intracameral, subconjunctival, or intravenous injections and topical application on the conjunctiva of 3 μL (30 μg) of cascade-blue–labeled Dextran (CB-Dx) were performed in Lewis rats. In addition, bilateral intracameral injections (CB-Dx into the right AC and FITC-Dx into the left AC) or a combination of intracameral (CB-Dx) and intravenous (FITC-Dx) injections were performed. Distribution of antigen-positive cells and free antigen in frozen sections of lymphoid organs from animals killed at 24 hours after these various types of injection was analyzed by fluorescence and confocal microscopy.

RESULTS. After intracameral and subconjunctival injections, antigen reaches the ipsilateral lymph node of the head and neck predominantly via the conjunctival lymphatics. Intraocular antigen entering the venous circulation reaches the spleen and mesenteric lymph nodes, but also a small proportion enters the lymph nodes of the head and neck. After bilateral intracameral injections, individual cells bearing both fluorescent antigens were identified in lymphoid organs draining the eye. Similarly, double antigen-positive marginal zone macrophages were observed after simultaneous intracameral injection of CB-Dx and intravenous injection of FITC-Dx.

CONCLUSIONS. These data demonstrate that in the first 24 hours after injection of antigen into the anterior chamber of the eye, antigen reaches the lymphoid organs mainly in a soluble form via both the blood and lymph. (Invest Ophthalmol Vis Sci. 2006;47:1039–1046) DOI:10.1167/iovs.05-1041

The nature of immune responses depends partly on the route of antigen (Ag) administration. A positive effector immune response (immunity) is induced after subcutaneous Ag immunization. In contrast, intracameral injection of Ag leads to a form of immune tolerance.1 The drainage pathways of soluble and cell-associated Ag between the skin and the draining lymph node (LN) after subcutaneous injection have been well characterized. A first wave of soluble Ag arrives in LNs via the lymphatics 90 minutes after Ag inoculation and distributes within LNs via the conduits formed by the reticular network before being internalized by interdigitating reticular network-associated dendritic cells (DCs).2,3 This first wave of Ag is followed 24 hours later by dermal DCs transporting a much higher concentration of Ag originating from the skin and is followed by a third wave of Ag transported by Langerhans cells another 24 hours later.4 By contrast, drainage pathways of Ag injected into the anterior chamber (AC) of the eye to the secondary lymphoid organs are not well defined.

Previous studies have shown that most of the Ag injected into the AC is filtered through the spleen.5,6 However, intracamerally injected Ag, intraocular tumor Ag, and Ag derived from corneal allograft also reach the LNs of the head and neck7–13 and the mesenteric LNs (MLNs).13 Although it is known that the drainage pathways of Ag from the AC to the spleen follow the conventional outflow pathway of aqueous humor to the venous system,14,15 the exact drainage pathway of Ag from the eye to the LNs of the head and neck and to the MLNs is unknown. In particular, the role of conjunctival lymphatics in this egress of Ag has not been determined.

It has been generally accepted that Ag originating from the AC of the eye is transported by ocular antigen-presenting cells (APCs).1,10,16–18 Analyses of the fate of Ag injected into the AC indeed indicate that Ag is captured predominantly by macrophages, both in the iris and subconjunctival connective tissue, which can be observed as early as 6 hours to 12 days after exposure to Ag.19–21 However, transportation of Ag by such cells to the lymphoid organs has never been directly observed. We have reported that Ag injected into the AC of the eye is localized in cells located in the subcapsular sinus and expressing CD1, CD4, CD8 (some cells), CD11b, CD68, CD86, CD163, CD169, and CD172 and in cells in the marginal zone (MZ) of the spleen expressing CD1, CD11b, CD68, CD86, CD163, and CD169.13 These phenotypes correspond exactly to the marker expression reported previously on subcapsular sinus macrophages and MZ macrophages, respectively.22 In particular, CD169 (sialoadhesin), is a specific marker for these populations, and therefore we used the anti-CD169 mAb (ED5) to detect subcapsular and MZ macrophages throughout the study. Localization of intracamerally injected Ag within these resident macrophages of the secondary lymphoid organs13 and emerging evidence that iris-derived APCs may lack the ability to migrate from the eye23 suggest that Ag may travel from the eye in a soluble form.

The purposes of the present study were therefore to determine Ag drainage pathways from the AC of the eye to the LNs of the head and neck, the spleen, and the MLNs, by comparing Ag distribution in the secondary lymphoid organs after intracameral, subconjunctival, and intravenous injection or topical application of Ag. These data, together, with bilateral ocular injections and combined intravenous and intracameral injections would hopefully help us to determine whether Ag placed into the AC of the eye travels to the secondary lymphoid organs in a soluble or cell-associated form.
**Materials and Methods**

**Animals**

Female Lewis rats, 8 to 11 weeks old, were obtained from the Animal Resources Center (Murdoch University, Western Australia) and kept under pathogen-free conditions in chaff-lined cages and housed in 12-hour day–night light cycles. Food (Stockfeeders RM2 autoclaved rat and mouse diet; Animal Resources Center, Western Australia) and water were supplied ad libitum. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and The University of Western Australia animal welfare regulations.

**Reagents and Antibodies**

Chemical reagents and antibodies were purchased from the following companies: bovine serum albumin (BSA), paraformaldehyde, and pentobarbinte sodium from Rhone Merieux (Queensland, Australia); purified monoclonal antibody (mAb) anti-dinitrophenyl (DNP, clone SPE-7) from Sigma-Aldrich (St. Louis, MO); purified mAb anti-sialoadhesin, purified monoclonal antibody (mAb) anti-dinitrophenyl (DNP, clone SPE-7) from Sigma-Aldrich (St. Louis, MO); purified mAb anti-sialoadhesin, and mouse diet; Animal Resources Center, Western Australia) and kept under pathogen-free conditions in chaff-lined cages and housed in 12-hour day–night light cycles. Food (Stockfeeders RM2 autoclaved rat and mouse diet; Animal Resources Center, Western Australia) and water were supplied ad libitum. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and The University of Western Australia animal welfare regulations.

**Ag Injections**

During all injection procedures animals were anesthetized by oxygen and nitrous oxide (4:1) and 1.0% halothane (ICI Pharmaceuticals, Grand Island, NY) was injected into the AC. Leakage of Ag was minimized by introducing a small air bubble into the AC. Animals that received a single AC injection comprised group I (Table 1). In animals that received a subconjunctival injection, 5 μL (30 μg) of CB-Dx (group II), the Ag was placed deep to the bulbar conjunctiva in the inferior quadrant of the right eye using identical fine glass microcannulae, raising a small conjunctival bleb. In group III, Ag (3 μL, CB-Dx) was applied topically to the surface of the right cornea and conjunctiva. In group IV, animals received a single intravenous injection, consisting of 30 μg CB-Dx or FITC-Dx diluted in 300 μL of sterile PBS, via a tail vein with a 25-gauge butterfly needle (Terumo, Tokyo Japan). In group V, animals received an injection of CB-Dx into the right AC and FITC-Dx into the left AC. In group VI, animals received an injection of CB-Dx (3 μL) into the right AC and a concurrent intravenous injection of FITC-Dx (diluted in 300 μL of sterile PBS). Details of the number of animals and tissues analyzed are shown in Table 1.

**Tissue Collection and Processing**

One day (24 hours) after Ag injection, animals were euthanatized by intraperitoneal injection of pentobarbinte sodium (100 mg/kg body weight) and the following secondary lymphoid organs (named according to the nomenclature of Tilney24) were collected (Fig. 1A): right and left submandibular LNs, superficial cervical, facial, deep cervical (also referred to as internal jugular nodes), brachial, axillary, and inguinal LNs; MLNs; and spleen. In addition hemolymph nodes: namely parathymic, mediastinal, and splenic were collected (Fig. 1A). All tissues were postfixed in 4% paraformaldehyde overnight before they were embedded in optimal cutting temperature (OCT) compound and stored at ~80 °C. Frozen sections (8 μm thick) were cut with a cryostat (CM 3050; Leica, Heidelberg, Germany). Sampling of lymphoid organs in the search for fluorescent Ag was performed on unstained sections by epifluorescence microscopy. The presence of Ag was readily dis-

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**Table 1. Distribution of Ag⁺ Cells in the Eyes and Secondary Lymphoid Organs after Various Types of Injections**

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II Subconjunctival Injection</th>
<th>Group III Topical Application</th>
<th>Group IV Intravenous Injection</th>
<th>Group V Bilateral AC Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac Injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Eye</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>6/6</td>
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<td>0/4</td>
<td>0/4</td>
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<td>0/5</td>
<td>0/5</td>
<td>0/4</td>
<td>0/4</td>
</tr>
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<td><strong>LNs of the head and neck</strong></td>
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</tr>
<tr>
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<td>5/6†</td>
<td>3/5</td>
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<td>2/4†</td>
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<tr>
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<td>2/5†</td>
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<tr>
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<td>4/4</td>
<td>5/5</td>
<td>0/3</td>
<td>1/4†</td>
</tr>
<tr>
<td>Left superficial cervical</td>
<td>2/5†</td>
<td>1/5†</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Right facial</td>
<td>3/5</td>
<td>5/5</td>
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<td>0/2</td>
</tr>
<tr>
<td>Left facial</td>
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<td>1/5†</td>
<td>0/2</td>
<td>1/2†</td>
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<tr>
<td>Right deep cervical</td>
<td>4/6</td>
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</tr>
<tr>
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<td><strong>Other somatic LNs</strong></td>
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<tr>
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<td>1/4†</td>
<td>0/4</td>
<td>0/4</td>
</tr>
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<td>0/3</td>
<td>0/4</td>
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<td>0/1</td>
<td>NT</td>
</tr>
<tr>
<td>Left axillaries</td>
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<td>0/2</td>
<td>0/1</td>
<td>NT</td>
</tr>
<tr>
<td>Right inguinal</td>
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<td>0/3</td>
<td>0/2</td>
<td>4/4</td>
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<tr>
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<td>0/3</td>
<td>0/3</td>
<td>4/4</td>
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<tr>
<td><strong>Hemo-LNs</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>0/2</td>
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<tr>
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<td>0/1</td>
<td>1/2</td>
</tr>
<tr>
<td>MLNs</td>
<td>6/6</td>
<td>2/5†</td>
<td>1/4†</td>
<td>2/4</td>
</tr>
<tr>
<td>Spleen</td>
<td>6/6</td>
<td>2/5†</td>
<td>0/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

*Data are tissues containing antigen (N positive/N tested. NT, not tested.*

* † n issues positive for cells bearing both CB-Dx and FITC-Dx/n tested.

† Present only in a few cells.
In each lymphoid organ or tissue studied, a minimum of four different depths were sampled, and five sections at each depth were cut for analysis. In the case of specimens in which Ag was difficult to detect, serial sections of the entire block were cut.

**Immunohistochemical Staining, Confocal Microscopy, and Image Analysis**

Epiluminescence microscopy (DMRBE; Leica) of sections was performed with long-distance 2.5 (N Plan) and 5, 10, 20, and 40 (PL Fluotar) objectives (Leica). Images were collected with a digital camera (model nDXM 1200F; Nikon, Tokyo, Japan) and software (ACT-1; Nikon). Confocal microscopy (model MRC-1000/1024 UV laser scanning confocal microscope; Bio-Rad, Hercules, CA) was used to characterize further the distribution of Ag and immunopositive cells. The microscope was equipped with argon lasers giving 351 nm (UV) and 488 nm (blue), and a helium-neon laser giving 543 nm (green), which allowed three fluorochromes to be observed. Sections were analyzed at either ×20 (Fluor 20× NA 0.75 objective; Nikon) or ×40 (Fluor 40× NA 1.3 oil immersion objective; Nikon), and sequential images were merged and pseudocolored (Confocal Assistant, ver. 4.02; freeware developed by Todd Clark Brelje and available at http://www.all-about-special-optics.com/resources/confocal-assistant-download.html) to produce a composite multicolor image. Final image processing was then performed (Adobe Photoshop; Mountain View, CA).

**RESULTS**

**Distribution of Ag in the Secondary Lymphoid Organs 24 Hours after Intracameral Injection: Group I**

After a single intracameral injection, fluorescent Ag was observed in CD169+ resident macrophages (red) in the subcapsular sinus of the right superficial cervical LNs (RSCLN), RSMLNs, and right deep cervical LNs (RDCLN). (D) Demonstration that leakage occurs from the corneal wound during an intracameral injection (right). Within seconds of withdrawal of the needle, patent blue V was detected in the inferior conjunctival fornix (left).

**FIGURE 1.** Drainage pathway after intracameral, subconjunctival inoculations and topical application of Ag. (A) Photographs of the secondary lymphoid organs studied. 1, right (ipsilateral) and, 2, left (contralateral) submandibular LNs at the upper pole of the maxillary glands; 3, right, and 4, left superficial cervical LNs; 5, right, and 6, left facial LNs at the junction between the facial veins and the external jugular veins; 7 right, and 8, left deep cervical LNs, also called the internal jugular LNs, lying deep to the lower pole of the maxillary glands and deep to the sternocleidomastoid muscle; 9, left mediastinal LNs; 10, spleen; 11, splenic LNs; and 12, MLNs. (B) Distribution of fluorescent Dx (white) in frozen sections of a variety of lymphoid tissues 24 hours after injection into the AC of the eye (group I). Note CB-Dx+ cells in the subcapsular sinus of the right submandibular LNs (RSMLN) and right superficial cervical LNs (SCLN), the medulla of the MLNs, and the MZ of the spleen. Cells containing FITC-Dx (40 kDa) were present in the subcapsular sinus of the mediastinal and splenic LNs. (C) Distribution of Ag (CB-Dx) in lymphoid tissues 24 hours after subconjunctival injection (group II). Large quantities of Ag were detected in CD169+ resident macrophages (red) in the subcapsular sinus of the right superficial cervical LNs (RSCLN), RSMLNs, and right deep cervical LNs (RDCLN). (D) Demonstration that leakage occurs from the corneal wound during an intracameral injection (right). Within seconds of withdrawal of the needle, patent blue V was detected in the inferior conjunctiva fornix (left).
the ipsilateral submandibular, deep and superficial cervical, and facial LNs (Fig. 1B), whereas Ag⁺ cells were more rarely observed in the contralateral LNs of the head and neck (Table 1). Fluorescent Ag was also detected in the subcapsular sinus macrophages of the MLNs, parathymic LNs (data not shown), splenic LNs and occasionally mediastinal hemolymph nodes (Fig. 1B, Table 1) and in MZ macrophages of the spleen (Fig. 1B).

Distribution of Ag in the Secondary Lymphoid Organs 24 Hours after a Subconjunctival Injection: Group II

Examination of lymphoid tissues from animals that received an injection of fluorescent Ag into the loose subconjunctival tissue 24 hours earlier revealed CD169⁺ Ag subcapsular sinus macrophages in the regional ipsilateral LNs and in a few cells in some contralateral LNs of the head and neck (Fig. 1C, Table 1). In two of five animals in this group, Ag⁺ cells were also present in the hemolymph nodes, the MZ of the spleen, and the medulla of the MLN (Table 1). This pattern of Ag distribution suggested that Ag drainage from the loose subconjunctival connective tissue was partly via the conjunctival lymphatics but also that some leakage into the venous circulation and other routes may have occurred. Thus, our data revealed that the nature and distribution of Ag⁺ cells in the lymphoid organs after subconjunctival and intracameral injection of Ag were equivalent (Table 1).

The Fate of Ag Leaking from the Corneal Wound during an Intracameral Injection: Group III

The presence of intracameral injected Ag in the subcapsular sinus and the medulla of the MLNs after intracameral injection prompted us to consider the possibility that some Ag injected into the AC may leak from the corneal wound onto the cornea and conjunctival surfaces. From there, it would be removed by the tears into the lacrimal apparatus, thereby reaching the mucosa of the nasopharynx, oropharynx, and presumably the remainder of the gastrointestinal tract, thus potentially allowing the draining MLNs. Because of the low visibility of the fluorescent tracers used in our experiment, leakage of Ag from the corneal wound site after its AC injection was not readily observable. Thus, to examine the possibility that undetected leakage may be occurring, 3 µL of the more visible dye, patent blue V, was injected into the right AC in a small group of rats (n = 3). This experiment followed protocols identical with that described for group I and that used by many investigators in the field during intracameral injections. Despite our usual precautions to avoid leakage, we observed traces of patent blue V exiting from the corneal wound onto the conjunctiva and accumulating in the fornix at the time of the injection and immediately afterward (Fig. 1D). This suggests that Ag may indeed gain access to the lacrimal drainage system and from there could reach the gastrointestinal tract.

To determine whether Ag leaking from the corneal wound reached secondary lymphoid organs, a group of animals (group III) received topical drops (3 µL) of CB-Dx onto the corneal-conjunctival surface. Investigation of the secondary lymphoid organs from these animals at 24 hours revealed a few Ag⁺ cells in the MLNs of one of the four animals, whereas all other lymphoid organs were devoid of Ag⁺ cells (Table 1). The quantity of Ag reaching the MLNs was considerably less than that observed after an intracameral injection. Moreover, we failed to observe fluorescent Ag in frozen sections of the oral cavity or trachea or in intestinal epithelial cells and/or M cells in the gastrointestinal tract (data not shown). These results suggest that the influence of the small quantity of Ag that may have leaked onto the ocular surface during intracameral injections on the distribution of Ag in the secondary lymphoid organs after intracameral injections was negligible.

Distribution of Ag in the Secondary Lymphoid Organs after an Intravenous Injection: Group IV

In the present study we sought to compare the distribution of Ag in secondary lymphoid organs injected into the AC (group I) with a single intravenous injection (group IV). After intravenous injection CB-Dx was present in the MZ of the spleen and in the subcapsular sinus of the hemolymph node, as expected. However, CD169⁺ macrophages bearing CB-Dx were also observed in the subcapsular sinus of the MLNs and some of the LNs of the head and neck, but in a less consistent pattern and in many fewer cells than were observed after intracameral injections (Table 1). Furthermore, the distribution of Ag to head and neck LNs was not asymmetrical, as was the case after intracameral injections. The occurrence of Ag in inguinal nodes was probably due to drainage from the tail via afferent lymphatics draining the tissues surrounding the venipuncture site.²⁴

These observations confirm that in the present experiments Ag detected in the spleen and hemolymph nodes after intracameral injection originated from the blood circulation and also indicate that blood-borne Ags reach the MLNs and, to a lesser degree, the regional LNs of the head and neck.

Entry of Ag Injected into the AC of the Eye into the Secondary Lymphoid Organs

In an attempt to elucidate the mechanisms by which Ag travels from the eye to the secondary lymphoid organs, we simultaneously injected CB-Dx (blue fluorescence) into the AC of the right eye and FITC-Dx (green fluorescence) into the AC of the left eye (group V). We postulated that if Ag exits the eye in a soluble form, it should be possible for CD169⁺ macrophages in draining LNs and spleen to internalize both CB-Dx and FITC-Dx. Alternatively, if Ag leaves the eye associated with ocular APCs, one would have expected that cells in draining LNs and spleen would be either CB-Dx⁺ (blue, derived from the right eye) or FITC-Dx⁺ (green, derived from the left eye) but not both.

Our analysis of lymphoid tissues revealed individual CD169⁺ cells bearing both fluorescent Ags in the MZ of the spleen (Fig. 2A) and the subcapsular sinus of the draining LNs (Fig. 2B, Table 1). Blue fluorescent Ag (CB-Dx) was the dominant form detected in the ipsilateral (right) submandibular, superficial cervical, and facial LNs, with a few of these cells also containing small quantities of green Ag. However on the left side (ipsilateral to eye injected with green Ag) the predominant type of Ag⁺ CD169⁺ cells were green with a few cells also containing small quantities of blue fluorescence. Similarly, both fluorescent Ags were present within the same cells in the MLNs (Fig 2D, bottom panel).

In search of evidence for the existence of ocular APCs bearing experimentally injected Ag, we performed a further double-fluorescent Ag experiment (group VI) in which CB-Dx (blue fluorescence) was injected into the AC of the right eye and FITC-Dx (green fluorescence) was injected immediately afterward directly into the venous circulation via the tail vein. If intraocular Ag exited the eye associated with ocular APCs, it seemed reasonable to predict that in the spleen, APCs would bear the blue Ag alone (CB-Dx⁺). Furthermore, the well-recognized resident cells in the MZ of the spleen, which receive blood-derived Ag,²⁵ would be predicted to be green only (FITC-Dx⁺). However, the results of the experiment revealed colocalization of both CB-Dx and FITC-Dx within the same macrophages in the spleen (Fig. 3). These observations suggest that, during the first 24 hours at least, the bulk of Ag injected...
into the AC of the eye left in a non–cell-associated form and was internalized by macrophages in the MZ of the spleen. Our previous data revealed a similar pattern of Ag distribution in the spleen at days 1, 3, 5, 7, and 12.

Although the distribution of 70-kDa CB-Dx injected into the AC of the eye was restricted to the subcapsular sinus macrophages of the LNs and in the spleen to the MZ macrophages, when A488-BSA (67 kDa) was substituted as the Ag, we noted that it served to highlight the reticular network of the right superficial cervical LNs (RFLN), right deep cervical LNs (RDCLN), left superficial cervical LNs (LSCLN), and the MLNs revealed they were CD169⁺ (red), indicating they were macrophages.

**DISCUSSION**

Until recently it was thought that Ag originating from the AC of the eye solely gained access via the blood to the spleen. It is now recognized, however, that intraocular derived Ag also reaches the LNs of the head and neck and the MLNs. There have been at least two previous studies of proliferation of Ag-specific lymphocytes after Ag placement in the AC, the subconjunctival sac, and the posterior chamber of the eye. In addition to confirming the basic pattern of Ag drainage into both LNs and spleen described in those proliferative studies, our data complement those by adding anatomic data not previously available. Our observations and morphologic demonstration that Ag injected into the AC of the eye also reached, in some cases, LNs that are not directly related to the lymphatic drainage system of the eye (i.e., the brachial, inguinal, and axillary LNs; Table 1, groups I and II) also correlated well with the data reported in the previous studies. Although immunofluorescent tracer molecules did not allow for the quantitation analysis of Ag in the various lymphoid organs, the technique was very sensitive and has allowed us to detect a minute amount of Ag in LNs that previously had not been thought to sample Ag derived from ocular tissues.

In the present study, we have further elucidated the drainage pathway from the AC of the eye to these lymphoid organs. Our present observations indicate that most of the Ag injected into the AC entered the venous circulation. This finding concurred with the well-accepted drainage mechanisms of aqueous humor from the AC via the conventional outflow pathway to the venous system. The present study also confirmed that the pattern of distribution of Ag to secondary lymphoid tissues after AC and intravenous injections bears strong similarities. However, our data also indicate that other pathways of Ag drainage exist between the AC of the eye and the...
secondary lymphoid organs—in particular, the LNs of the head and neck and the MLNs.

In the past, the absence of lymphatics within the eye appeared to support the hypothesis that there was no communication between the AC and regional LNs. However, several recent studies have indicated that the route of passage of Ag from the AC of the eye to the regional LNs may have been via the uveoscleral drainage pathway which communicates with the blood stream. Ag injected into the AC of the eye entered into the secondary lymphoid organs in a soluble form. After A488-BSA injection into the AC of the eye, Ag is internalized by resident CD169+ macrophages and distributes along the reticular fibers of the subcapsular sinus and high endothelial venules of the right superficial cervical LNs (RSCLN) and medulla of the MLNs. In the spleen, BSA was visible in the red pulp, the MZ, and surrounding the central artery in the white pulp.
the loose episcleral and subconjunctival tissues. Hoffmann et al. provided evidence of the existence of a drainage pathway in the mouse after intracameral and subconjunctival injections of radiolabeled colloidal albumin. Furthermore, in a previous study we demonstrated the presence of Ag-bearing macrophages and free Ag in the proximity of the nonconventional outflow pathways and episcleral tissues after an intracameral injection. We hypothesized that Ag from this region may have gained access to the lymphatics draining this loose subconjunctival connective tissue and thereby reached the subcapsular sinus of the ipsilateral draining LNs of the head and neck (submandibular, cervical, and facial LNs). The similar patterns of distribution of Ag to the secondary lymphoid organs after intracameral and subconjunctival injections further confirmed this hypothesis. After subconjunctival injections, Ag was also observed in the spleen and the hemolymph nodes, suggesting that some leakage into the venous circulation may occur from this loose subconjunctival connective tissue. The finding of a few Ag⁺ cells in some of the LNs of the head and neck after intravenous injection of Ag indicates that a small amount of Ag may have entered these LNs from the blood circulation as well as via afferent lymphatics. However, this would not explain the large quantity of Ag seen in these nodes after intracameral injections, which we believe was predominantly attributable to lymphatic drainage. The observations in the present study that Ag reaches LNs of the head and neck via both the lymphatic system and the intravenous route, however, concurs with previous observations that adoptively transferred CD4⁺ KI-J2-6⁺ OVA-specific T cells proliferate in the ipsilateral submandibular LNs in response to an intracameral injection and bilaterally after intravenous injection of OVA-derived peptides.

In the present study, as well as confirming the presence of Ag⁺ cells in the subcapsular sinus of LNs of the head and neck after intracameral injection, we also observed Ag⁺ cells in the subcapsular sinus of splenic, mediastinal, and parathymic hemolymph nodes. These hemolymph nodes filter erythrocytes or blood-borne Ag in lymph originating from the spleen, thymus, and other sites where erythrocytes pass into lymph in higher numbers than occurs in most regions of the body. The finding of only rare Ag⁺ cells in the MLNs of a minority of animals studied after experimental topical application of the same quantity of Ag onto the corneal surface suggested that leakage of Ag from the wound contributes to only a minor degree to the presence of Ag in the MLNs. The observation of Ag within the subcapsular sinus macrophages of the MLNs after tail vein injection of the same fluorescent Ag as used in our ocular studies concurs with previous reports and indicates that blood-borne Ag enters the MLNs. It has generally been accepted that Ag originating from the AC of the eye is transported by ocular APCs to the MZ of the spleen. Several pieces of evidence led us to postulate that Ag travels from the eye in a predominantly soluble form. These include the presence of fluorescent Ag derived from the AC within iris macrophages but not DC, the trapping of Ag by resident macrophages of the secondary lymphoid organs (as shown in the present study); and the recent data demonstrating the apparent inability of iris derived APCs to migrate from the eye. Two major experiments in the present study—notably, the bilateral intracameral injections of different colored fluorescent Ags and the simultaneous injections of CB-Dx in the right AC and FITC-Dx in the tail vein—sought to test this hypothesis. In both experiments, CD169⁺ macrophages containing both colors of fluorescent Ags were observed in the spleen and the draining LNs. This pattern of Ag distribution within the same cells in both these experiments supported the hypothesis that Ag exits the eye in a soluble or non–cell-associated form, at least transiently. The pattern of distribution of 67-kDa A488-BSA and 40-kDa FITC-Dx (data not shown) along the reticular fiber network of the LNs and spleen after injection into the AC of the eye was in accord with two independent previous descriptions of the distribution of the same soluble fluorescent Ag in draining LNs after subcutaneous injection and in the spleen after intravenous injection. The similarity in Ag distribution in the present study to these recent descriptions supports our hypothesis that, in the first 24 hours after injection, the bulk of Ag originating from the AC of the eye enters the secondary lymphoid organs in a non–cell-associated form. Our previous published study revealed that the pattern of Ag distribution in the secondary lymphoid organs was similar at 1, 3, 5, 7, or 12 days after intracameral injection. The report that as few as 20 F4/80⁺ Ag-bearing APCs may be sufficient to carry the tocoltyogenic signal from the eye to the blood to the spleen makes it unlikely that such a number of cells could ever be isolated by morphologic means alone. If this is the case, then it is hardly surprising that no evidence of such cells was found in the present study.

In summary, our results indicate that Ag injected into the AC of the eye reached the secondary lymphoid organs through several routes. First, the distribution predominantly to ipsilateral LNs of the head and neck illustrated that Ag drained partly via the afferent conjunctival lymphatics, a situation mimicked by injecting Ag directly into the loose subconjunctival connective tissue. Second, only small quantities of Ag that reached the MLNs could be attributed to leakage from the corneal wound and drainage via the lacrimal system. Third, our data strongly indicate that Ag when placed into the AC of the eye reached many peripheral non-draining secondary lymphoid organs (e.g., MLNs, hemolymph nodes, spleen) via the venous circulation and that the pattern of Ag distribution can be partially replicated by intravenous injection. Furthermore, our bilateral ocular injections of different colored fluorescent Ags taken together with the concomitant intracameral and intravenous injections lent strong support to the hypothesis that bulk Ag drainage occurs predominantly in a non–cell-associated form. The pattern of distribution of BSA and 40-kDa Dx along the reticular network in the secondary lymphoid organs after intracameral injection is similar to that described by others after subcutaneous and intravenous injections. Therefore, these observations gave strong support to the concept that the bulk of Ag exiting the AC of the eye travels in a non–cell-associated form. In conclusion, our experiments demonstrated that both lymphatics and vascular routes acted as major means of afferent intraocular Ag access to lymphoid tissues.

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References


