Lymphatic drainage of $^{131}$I-albumin from the vascularized cornea

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The presence of lymphatic vessels in the vascularized rabbit cornea has been demonstrated previously. In this paper it is shown that an $^{131}$I-albumin-Evans blue complex injected into the center of a lymph-vascularized rat cornea may be carried to the lymph nodes of the neck within six minutes. This time corresponds to the time required for the same inoculum to be carried from the bulbar conjunctiva, a lymphatic-rich tissue, to the same lymph nodes. Moreover it is shown that although $^{131}$I-albumin can pass through the normal or edematous corneas by a process of diffusion this passage is slow in that the time taken from cornea to lymph nodes is about six hours and the radioactivity in the nodes may be only one thousandth of that found with vascularized corneas. These findings are considered as evidence on a functional level that lymphatic vessels, when present in a vascularized mammalian cornea, are channels of rapid movement of other similar-sized molecules in the cornea, including antigens from a grafted cornea.

Key words: lymphatic drainage, corneal vascularization, corneal edema, albumin, Evans blue, iodine isotopes, time factors, lymph nodes, neck, corneal graft, graft rejection, rats.

With the recognition of corneal graft rejection as an immunological phenomenon the movement of substances of large molecular weight, particularly proteins, through the cornea has become of specific clinical interest in the understanding of these immune reactions. The diffusion kinetics in relation to the movement of injected foreign protein in the cornea of rabbits has been investigated by Sery and his collaborators using equine albumin and by Maurice and Watson using rabbit albumin. In general, the results are similar; that is, they found a transfer coefficient of 0.21 to 0.25 per day or 0.18 to 0.29 per day and a diffusion constant of $1.1 \times 10^{-7}$ cm$^2$ per second. However, in all of these investigations the corneas were normal or, at least, not vascularized. It is known that the presence of marked blood vascularization of the recipient cornea constitutes a serious contraindication for keratoplasty and hence the passage of protein through a vascularized cornea warrants investigation.

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Table I

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Condition of cornea at time of injection of $^{131}$I-albumin</th>
<th>Means of production of corneal damage</th>
<th>Period over which damage was produced</th>
<th>Site of injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cornea</td>
<td>13</td>
<td>Normal</td>
<td>—</td>
<td>—</td>
<td>Center of cornea (stroma)</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>11</td>
<td>Normal</td>
<td>—</td>
<td>—</td>
<td>Conjunctiva</td>
</tr>
<tr>
<td>Anterior chamber</td>
<td>7</td>
<td>Normal</td>
<td>—</td>
<td>—</td>
<td>Anterior chamber through the center of the cornea</td>
</tr>
<tr>
<td>Conjunctival sac</td>
<td>4</td>
<td>Normal</td>
<td>—</td>
<td>—</td>
<td>Conjunctival sac with lids sutured together</td>
</tr>
<tr>
<td>Edematous cornea</td>
<td>7</td>
<td>Edematous (not vascularized)</td>
<td>Injection of 5 $\mu$l of 0.1N hydrochloric acid into central corneal stroma</td>
<td>18 to 24 hr.</td>
<td>Center of cornea (stroma)</td>
</tr>
<tr>
<td>Vascularized cornea</td>
<td>15</td>
<td>Vascularized with some edema</td>
<td>Injection of 5 $\mu$l of 0.1N hydrochloric acid into central corneal stroma</td>
<td>10 to 22 days</td>
<td>Center of cornea (stroma)</td>
</tr>
<tr>
<td>(short-term)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascularized cornea</td>
<td>22</td>
<td>Vascularized with no edema</td>
<td>Injection of 5 $\mu$l of 0.1N hydrochloric acid into central corneal stroma</td>
<td>42 to 96 days</td>
<td>Center of cornea (stroma)</td>
</tr>
<tr>
<td>(long-term)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It was the purpose of this study to examine the movement of human serum albumin from the cornea to the neck lymph nodes in mammals under various pathological conditions and to ascertain whether a functional and direct route is available in the vascularized cornea and if this is connected directly with the normal lymphatic drainage of the eye as has been claimed previously.1, 2, 16

Methods

Adult albino rats of either sex, weighing between 200 and 300 grams, were used throughout this series of experiments. In all, 82 rats were used. However, of these, one died during the experiment, one had a vascularized cornea of unknown origin, and in one of the conjunctival sac series the technique of injection was faulty. The remaining 79 rats were treated as follows.

All injections were made under ether anesthesia.

With the use of a 30 gauge needle attached to a Hamilton Teflon-tipped 50 $\mu$l microsyringe, working under a Zeiss binocular dissecting microscope, 5 $\mu$l of $^{131}$I-radioiodinated human serum albumin B.P.4 plus Evans blue was injected into various sites in the normal and pathological left eyes of all rats as shown in Table I.

In the case of corneal injections the actual depth of the injection was unknown, although all injections were within the stroma and not merely below the epithelium. The injection produced a blue blob which was approximately 3 mm. in diameter in the center of the cornea. Thus a 2 mm. annulus of clear cornea surrounded the injected material.

Prior to the injection of $^{131}$I-albumin, all eyes were carefully examined with the biomicroscope. The corneas of the edematous and short-term vascularized groups showed central edema, whereas those of the long-term vascularized group showed slight central opacification (nebulae). Except for the blood vessels, the peripheral cornea of both vascularized groups was transparent. The neighboring bulbar conjunctiva appeared normal and there was no evidence of a conjunctival invasion of the cornea. This was confirmed by histological sections of the cornea.

The rats were permitted to revive and, at various intervals from 5 minutes to 29 hours, the animals were killed by an overdose of ether. Eight sets of lymph nodes (four from each side) were then removed from each rat. When dissecting from the ventral side, these were found (1) anterior to the submaxillary gland, (2) anterior to the parotid gland, (3) below the parotid gland,
and (4) below the sternocleidomastoid and omohyoid muscles. These nodes were selected because of their ease of access and removal and because they are sufficiently distant from the site of injection to eliminate the possibility of diffusion from the site of injection to the nodes. Also, Bill has shown in rabbits that albumin diffusing from the suprachoroid was drained predominantly to the submaxillary and superficial and deep cervical lymph nodes. It is difficult to name each lymph node, for of the rats at least 50 per cent had more than one node in each of the four sites mentioned. The extreme variability made it advisable to standardize on four node sites rather than a variable number (from 4 to 9) of individual nodes.

The nodes were placed in nylon containers and the activity of each group of nodes was measured by means of a well crystal and an EKCO scintillation counter (N530D) set at 1100 volts and a discriminator bias of 15 volts. Each sample was counted for 100 seconds. The background count was found to be 0.88 counts per second (S.D. ± 0.10). However, the background count is not shown in any of the results as presented here.

In addition, in the majority of animals the cornea plus part of the anterior sclera of the injected eye was removed and the radioactivity was assessed in a similar manner.

Mathematical treatment of results. The radioactive solution used for injection consisted of 0.220 to 0.224 ml. of 131I-radioiodinated human serum albumin containing 4.13 ± 0.01 mg. of albumin and having 0.2 millicurie of activity at a specified time. To this solution was added concentrated Evans blue solution in sterile isotonic saline to make the total volume 0.30 ml.

After being counted, all results were corrected to the specified time according to the iodine-131 correction table.

The activity of each of the eight nodes (or sites) from each rat was counted for a total of 100 seconds. The results for the four nodes from the right or noninjected side of the animal were averaged (R mean). This figure was then subtracted from the count obtained for each of the nodes from the left or injected side of the animal (Lx). Thus, four results were obtained for each animal (Lx−R mean).

All results were analyzed statistically using Student's t test.

In the early stages lymph nodes were weighed to ascertain whether apparently significant results were merely the result of increased lymph space in the large nodes. However, it was soon evident that there was no direct relationship between the count and the node size and this procedure was discontinued.

Results

Corneas. In all corneas there was a very fast decline of radioactivity with time (see Fig. 1). In particular, in that group of animals in which the corneas were made edematous prior to the injection of 131I-albumin, the fall in count was very marked in the initial period, reaching below 500 counts per second after only about two hours, whereas in the normal corneas this level is reached only after about twenty hours.

In the corneas which have been vascularized over a period of 10 to 22 days and in which there is still some remaining corneal...
edema, the activity again falls very quickly and reaches the 500 counts per second level after about four hours. As can be seen in Fig. 1, the rate of loss of activity is almost identical with that shown for edematous corneas.

When we consider those corneas which have been undergoing vascularization for from 40 to 96 days and the edema is either absent or minimal, the rate of decrease of activity is less than that found in both sets of edematous corneas but considerably greater than that for normal corneas. The level of 500 counts per second is reached somewhere between 7 and 12 hours after injection.

**Lymph nodes.** Fig. 2 shows the distribution of the radioactivity in the lymph nodes (Lx–R mean) removed from those animals in which the isotope-protein complex was injected into the edematous cornea, the anterior chamber, or the conjunctival sac.

In the first of these groups, three nodes out of 28 were found to be significantly higher than the mean of the nodes on the noninjected side (significant at the 0.001 level) whereas in the second group only one high count (significant at the 0.005 level) out of the 28 was found, and in the third group no result out of the 16 nodes was significant.

The lymph node count from the fourth negative control group consisting of normal corneas is shown in Fig. 3 (note that the horizontal scale in steps of 10 counts per second for Figs. 3 and 4 is one twentieth of that for Figs. 2 and 5, in which the steps are 0.5 count per second each). When this scale is used, no nodes fall outside the first two ranges of plus or minus 10 counts per second, although 8 nodes out of 52 are significantly higher than R mean at the 0.001 level.

The positive control group in which the injection was made into the lymphatic-rich conjunctival tissue adjacent to the cornea gave a large number of significant results and these too are shown in Fig. 3. Again on this scale only 9 out of 44 nodes have counts of greater than 10 per second (Lx–R

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**Fig. 2.** Distribution of excess of radioactivity of the lymph nodes on the injected side of the animal (Lx–R mean, see sext) in three of the negative control groups. The numbers in brackets represent the total number of left nodes for each group, that is, four from each animal.

**Fig. 3.** Distribution of excess of activity of left lymph nodes (Lx–R mean) following injection of 131I-albumin into the normal left corneas (negative control) and into the conjunctiva (positive control). Note the change in scale of Figs. 3 and 4 compared with Figs. 2 and 5.
mean greater than 10). However, there are 23 of the 44 nodes with a value significantly higher than R mean at the 0.001 level.

In this group it was also found that there were marked differences in the results obtained from animals killed after very short periods. If the rat was killed prior to its awakening from the anesthesia and hence prior to any activity, a very low count was obtained, whereas if the rat revived, began to move about, and was then killed—a minimum of about six minutes after injection—a much higher count resulted. This effect is related to the increase in lymph flow which results from muscular activity.

In the group of rats in which one eye had been undergoing vascularization for 10 to 22 days, only one lymph node out of 60 has a count (Lx–R mean) greater than 10 per second, whereas in the group vascularized over 40 to 96 days there are 16 out of 88 nodes (Fig. 4). The proportion of high counts in the long-vascularized group (16 of 88 above 10 per second and 34 of 88 significantly greater than R mean at the 0.001 level or 43 of 88 significant at the 0.005 level) is similar to that recorded for the positive control group of injected conjunctivas, that is, 9 out of 44 with Lx–R mean above 10 counts per second and 23 of 44 significantly greater than R mean at the 0.001 level or 26 of 44 significant at the 0.005 level.

However, the findings for the short-term vascularized rats are not as insignificant as they appear in Fig. 4. First, there are 18 out of 60 nodes in which the Lx–R mean value is significant at the 0.001 level (compared with 8 of 52 for the normals). Second, if we compare the short-term vascularized corneas and the normal corneas on a scale twenty times that of Fig. 4, and divide the results into two groups representing periods of zero to 5 hours and 5 to 30 hours for the time from the injection to the death of the animals, further facts become apparent (Fig. 5).

In the normal rats no counts greater than 0.5 per second were found for injection times less than five hours, while eight nodes had counts between 0.5 and 2.0 per second when the animals lived for longer than five hours after the injection. By contrast, 12 out of 36 of the nodes removed from animals with vascularized corneas (10 to 22 days) after less than five hours had counts ranging from 0.5 to 11.1 counts per second, while only 5 out of 24 nodes with counts (Lx–R mean) ranging from 0.5 to 1.5 per second were found in the 5 to 30 hour time range.

This marked difference in the magnitude of the measured activity between nodes
Lymphatic drainage of ¹³¹I-albumin

from animals with normal or vascularized corneas is further illustrated in Fig. 6, in which the node counts (Lx-R mean values) from normal, edematous, and both early and late vascularized corneas are plotted on a logarithmic scale against the drainage time after injection.

With the normal animals the node count gradually increases to a significant level, e.g., 1.0 count per second at about 5 to 6 hours. The nodes from the animals with edematous corneas have a slightly higher count than the normals in the short-term injections, but otherwise have a very similar distribution.

For the nodes from animals with vascularized corneas the highest counts are obtained for the very short times of injection, e.g., up to a half hour. Some very high counts resulted only six minutes after injection. After this time the activity decreases quickly from 1,580 counts to about 16 counts per second at four hours after injection.

Discussion

The rate of removal of ¹³¹I-albumin from the cornea, particularly the nonvascularized cornea, as shown in Fig. 1, is far greater than can be accounted for by either diffusion or lymphatic drainage. In fact, the major route of exit is via the gap in the surface of the cornea resulting from the insertion of the needle through which the protein was injected. Sery, Pinkes, and Nagy⁹ have briefly investigated this loss of injected albumin via the needle track and found that only 49 to 87 per cent of the injected material (an average of 71 per cent) is retained initially. After as little as three hours, this level has further decreased to as low as 14 to 37 per cent of the intended inoculum. Sery worked with rabbit corneas, injecting 20 μl. As the volume of the rat cornea is approximately one fifth of that of the rabbit, the injection of 5 μl into the rat cornea as used in this investigation should give similar findings.

It is also evident that, in those corneas which are edematous but not vascularized, the increased rate of loss is primarily the result of diffusion, which is greatly facilitated in the edematous condition, and hence the ¹³¹I-albumin reaches the surface puncture more readily and leaks out. Although there are some significant lymph node counts with edematous corneas (Fig. 2), these are less than for the normals (Fig. 3). The more rapid loss from the edematous cornea reduces the concentration of ¹³¹I-albumin and thus reduces the diffusion rate.

There is no significant difference between the loss from the cornea in the edematous group and that in which the corneas are recently vascularized, but the rate of removal of ¹³¹I-albumin from the markedly vascularized group in which there is no edema is less than that for the edematous
Fig. 6. The excess of activity of the left lymph nodes (Lx–R mean) of the normal, edematous, and vascularized experimental groups shown in relation to the time between injection and death of the animal. Observe the very high counts for very short times in the vascularized groups and the slow increase in count for normal and edematous groups (note the logarithmic scale).

groups but greater than that for the normals. This may reflect the degree of lymphatic drainage which takes place in these vascularized corneas.

The findings for the lymph nodes from the control group of animals receiving an anterior chamber injection indicate that very little protein from the cornea will reach the nodes by passing through the corneal endothelium and thence leaving the eye via the conventional outflow paths or by way of the uveoscleral routes which
are available. Similarly, although there is a considerable loss of injected protein through the needle track, the results from the "conjunctival sac" group of animals show that this escaped protein cannot penetrate the conjunctival epithelium and so gain access to the conjunctival lymphatics and the nodes.

The finding that $^{131}$I-albumin diffuses through the corneal stroma to the limbus and thence enters the conjunctival lymphatics and is carried to the lymph nodes in the neck confirms the claims that the pericorneal lymphatics can be filled as a result of intracorneal injection. These workers injected an asphalt-chloroform solution and India ink but attempts to reproduce their results using a Mandarin ink were unsuccessful. The sensitivity of this method using a radioisotope is far greater than the purely visual methods previously employed.

That albumin (M.W. 69,000) diffuses through the normal cornea is not a new finding. It has been extensively investigated by Maurice and Watson and Maurice claims that the limiting size for particles which will diffuse through the normal cornea corresponds to a molecular weight of around 500,000.

In this investigation, if the concentration of albumin at the limbus is assumed to be proportional to the total node count and that at the center of the cornea to be proportional to the total count injected, a ratio of concentrations can be obtained.

If the finding of Sery, Pinkes and Nagy that within three hours as little as 14 per cent of the injected protein remains in the cornea is considered in conjunction with the results shown in Fig. 1, a reasonable estimate of the average ratio of concentrations between the center of the cornea and the lymph node count is 500 to 1.

The expression of Maurice and Watson:

\[
\text{Concentration ratio} = e^{-\frac{p^2}{4Dt}}
\]

where \( p \) is the distance between the two points, \( D \) is the diffusion constant, and \( t \) is the time taken for the diffusion to take place, can be used to describe the rate of diffusion through the cornea. Inserting the time of five hours as the minimum time found for a significant quantity of radioactivity to appear in the nodes, and using the figure of 2 mm. as the distance in the cornea through which the albumin must diffuse to the limbus, the calculated value of the diffusion constant is $0.90 \times 10^{-7}$ cm.$^2$ per second.

In view of the numerous assumptions made, this figure compares favorably with that of Maurice and Watson, that is, $1.1 \times 10^{-7}$ cm.$^2$ per second. Thus it is reasonable to assume that the positive and significant lymph node counts recorded in normal animals at times greater than five hours are the result of diffusion.

The fact that the highest node activity is observed after times as short as six minutes is evidence that the movement of albumin in vascularized corneas is not the result of diffusion. Also, the time taken is consistent with the findings for the positive control group of conjunctival injections and with other investigations, such as that of Mayerson, who found a ten-minute delay between the time of infusion of labeled albumin into a leg lymphatic duct of the dog and its appearance in the thoracic duct. Thus it can be assumed that there is a direct pathway from the central region of these vascularized corneas to the lymphatics of the conjunctiva and thence to the lymph nodes. That this pathway is in fact an extension of the conjunctival lymphatic system in the form of endothelial-lined lymphatic vessels has already been presented.

The conjunctiva is known to be very richly supplied with lymphatic vessels. As the number of nodes with high activity is similar for the group receiving conjunctival injections and for that vascularized over a long period, it follows that the lymphatic network in the vascularized cornea may be as extensive as that in the normal conjunctiva.

This facilitation of movement of protein
due to the presence of lymphatics in the cornea leads to the assumption that the passage of antigens from donor corneal grafts will similarly be assisted by the presence of corneal lymphatics. It is claimed both that graft antigens can diffuse through the cornea and that blood or lymphatic vessels are not necessary for the sensitization of a host. However, there is little doubt that the passage of these particles through the cornea would be facilitated by the presence of lymphatics, particularly if the antigens are larger than the 500,000 molecular weight, which may be the largest size of particle which can move through the normal cornea. It is of course recognized that the presence of corneal edema may permit the passage of large particles which would otherwise be stationary. However, from this study it can be seen that the effect of edema is far less than that due to corneal lymphatics.

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REFERENCES

Lymphatic drainage of $^{131}$-albumin


