Lymphocyte-induced corneal neovascularization: a morphologic assessment

Randy J. Epstein and W. Franklin Hughes

Lymphocytes obtained from mesenteric lymph nodes of rabbits were stimulated in vitro by concanavalin A and injected into the corneas of allogeneic hosts. Controls were nonstimulated, killed, or autogeneic lymphocytes injected into the contralateral corneas. The stimulated cells were significantly better inducers of neovascular growth. Histologic observations of the vascularized corneas showing marked mononuclear reactions at the limbus, coupled with the requirement of allogenicity, suggest that there was immunologic recognition of the implanted cells. Stimulated allogeneic blast cells may amplify this host recognition by their elaboration of lymphokines or enhanced antigenicity. The apparent importance of allogenicity for the induction of vessel growth in these experiments may be significant in the pathogenesis of tumor-induced or graft-related corneal neovascularization.

Key words: stimulated lymphocytes, concanavalin A, corneal neovascularization, inflammation, angiogenesis

Vascular invasion of the cornea is important clinically and is the subject of active experimental investigation. The neovascular growth is accompanied in many instances by infiltration of the stroma with polymorphonuclear leukocytes (PMNs), although it is still not clear whether they initiate these reactions or serve a facilitative role. In contrast, neovascularization resulting from hetero
genetic tumor implants has been interpreted as having occurred without the initiation of inflammatory or immune responses. There is little uniformity of opinion regarding a common neovascular stimulus or the role that various cell types play in initiating or sustaining capillary growth.

Recently evidence has been accumulating that favors the hypothesis that many forms of corneal neovascularization involve inflammatory or immune responses. Tumor angiogenesis factor (TAF) has been biochemically characterized and has been found to provoke an impressive inflammatory response in one system. Lymphocyte-induced angiogenesis was introduced as a quantitative and sensitive measure of the graft vs. host reaction by Sidky and Auerbach, who noted its striking similarity to tumor-induced neovascularization. Using the chick chorioallantoic membrane (CAM), Fliskin et al. showed that lymphocytes stimulated with phytohemagglutinin (PHA) were angiogenic.
found that although lymphocytes stimulated by concanavalin A (Con A) were angiogenic in the cornea, PHA-stimulated lymphocytes were not. Recently an extract with lymphokine-like activity derived from the seastar has been found to be angiogenic on the CAM. 17

The present study was undertaken to evaluate histologic features of the neovascular response to Con A-activated lymphocytes in an attempt to determine the importance of the host response and to examine some of the variables that might be important in the angiogenic process.

**Methods**

Lymph node dissections and corneal injections were performed on nonlittermate New Zealand White and Red rabbits (2 to 3 kg) under general anesthesia (ketamine 50 mg/kg and acepromazine 10 mg/kg, intramuscularly).

**Lymphocyte preparation.** In vitro stimulation of lymphocytes was modified from Sheppard et al. 18 Mesenteric lymph nodes were dissected from live rabbits by strict sterile technique. These were suspended in medium RPMI 1640, filtered through No. 40 mesh, subjected to hypotonic lysis with distilled water to destroy red blood cells, and filtered again through No. 80 mesh. The cells were brought to a concentration of 2 X 10^6 cells/ml in medium supplemented with 10% to 15% autologous serum, 20 mM HEPES buffer, 100 mg/ml streptomycin, and 100 IU/ml penicillin. To half of these cells, Con A (Pharmacia) was added in a final concentration of 10 /ng/ml; the remainder served as controls. Aliquots (0.2 ml) of these suspensions were incubated in flat-bottomed microtiter plates at 37° C with 5% CO₂ and 98% room air for 48 to 72 hr. The cells were harvested and washed in fresh medium without Con A. The concentration of viable cells, determined by trypan blue dye exclusion with a hemocytometer, was adjusted to 10^7 to 10^8 cells/ml.

Stimulation assays, as described by Meo, 19 were performed on test cultures that received ^3H-thymidine (0.5 μCi/well) 12 hr prior to termination of the culture period, and were processed in a sample harvester for scintillation counting. A stimulation index (SI) was calculated with the formula:

$$SI = \frac{CPM \text{ stimulated}}{CPM \text{ unstimulated}}$$

**Corneal injections.** As others have found,11, 13 about 10^6 stimulated cells per injection were necessary for the induction of neovascular responses. Midstromal injections of 10^6 cells in 5 μl were made 3 to 4 mm from the superior border of the cornea so that the cells spread to within 2 to 2.5 mm of the limbus. Each rabbit received an injection of stimulated cells in one cornea and control cells in the other. Control injections included the following: autogeneic stimulated lymphocytes, allogeneic lymphocytes cultured without Con A, allogeneic lymphocytes that were stimulated and then killed by freeze-thawing or puromycin treatment, and injections of equal volumes of saline and latex microspheres. Additional controls included both autogeneic and allogeneic lymphocytes that were isolated and injected without culturing.

All injections and subsequent assessments were performed in a double-blind format. Observations were also made independently by two observers.

**Histologic examination.** Corneas from each experimental group were selected for histologic study at intervals between 24 hr and 4 weeks. Specimens were prepared so that each portion contained both the injection site and the adjacent limbus. One portion was fixed in buffered 10% formalin and processed routinely for light microscopy; the other was prepared for electron microscopy by fixation in 1% paraformaldehyde-2% glutaraldehyde in cacodylate buffer (pH 7.5), postfixed in osmium tetroxide, and embedded in a low-viscosity resin for thin sectioning.

Selected animals received 50 μCi subconjunctival injections of ^3H-thymidine (6.7 Ci/mmol) 6 hr prior to sacrifice in preparation for radioautographic study of the corneas. Paraffin was removed from sections of this material, which were then coated with NTB-2 emulsion and exposed for 3 to 4 weeks prior to development and staining.

---

**Table I. Corneas with vessels ≥ 1 mm at 8 days**

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Corneas vascularized/total</th>
<th>% Vascularized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A—stimulated</td>
<td>33/69</td>
<td>48</td>
</tr>
<tr>
<td>(allogeneic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonstimulated (allogeneic)</td>
<td>11/69</td>
<td>16</td>
</tr>
<tr>
<td>Stimulated/killed (allogeneic)</td>
<td>0/12</td>
<td>0</td>
</tr>
<tr>
<td>Autogeneic/stimulated</td>
<td>0/8</td>
<td>0</td>
</tr>
</tbody>
</table>

---
Results

A positive response was defined by the appearance of at least 1 mm of neovascular outgrowth within 8 days of the injection. The results of 12 separate experiments are summarized in Table I. Corneas injected with stimulated cells met the criterion for a positive neovascular response in 33 of 60 (45%) of the cases. Unstimulated cells produced neovascular responses in only 11 of 69 (16%) of the corneas. The difference between these two groups is significant at the p < 0.001 level ($\chi^2 = 52.34$, 3 df). No reactions occurred after injections of autologous or killed cells that had been stimulated by Con A. Finally, control cells cultured without Con A, cells implanted immediately without culture, and latex microspheres showed no significant angiogenic potential.

Neovascular responses obtained from unstimulated control cells were generally accompanied by a positive response to stimulated cells in the contralateral cornea, suggesting that these rabbits were strong responders. In some instances, the lack of neovascularization after injections of stimulated cells accompanied experiments where the stimulation index was unusually low.

Gross responses. Within 24 hr after injection, limbal hyperemia and vascular engorgement were noted. This persisted in those eyes that proceeded to vascularize, but subsided within 72 hr in those that did not. In the positive responses, vessels could be seen invading the corneal stroma from 3 to 5 days after the injection (Fig. 1, a). These corneas showed a white fluffiness at the injection site, but no stromal edema was evident between the limbus and injection site. By the sixth day, vessels had formed a small band, 5 mm wide, which had reached at least 1 mm in length. By 10 to 15 days after injection, most of the marginal vessels had regressed, leaving only a few direct channels to the in-
Fig. 2. Stimulated lymphocytes at the injection site. a, Lymphocyte injection site (L) is seen at 72 hr, with some PMNs in the superficial stroma (arrows). (×295.) Inset, Thymidine uptake is still evident in the injected cells. (×990.) b, Injected lymphoblasts (L) appear viable at 72 hr. (×13,788.) c, Injection site at 8 days shows mainly pyknotic nuclei (N) and cellular debris. (×5294.)
Injection site (Fig. 1, b). Involution of the vessels by 4 weeks left no visible signs.

**Histologic observations.** Light and electron microscopic observations revealed the following pattern of response.

**Injection sites.** At 3 days, the sites injected with stimulated cells showed intact lymphoblasts (Fig. 2, a). The viability of the cells was confirmed by radioautography, which showed that the stimulated cells were still undergoing mitosis at this time (Fig. 2, a, inset), in contrast to the control cells, which were not. Electron microscopy also showed that the stimulated cells (Fig. 2, b) had retained their viability better than cells at the control injection sites, where signs of degeneration were evident. By 8 days, however, the uptake of label at all injection sites had ceased and cellular debris predominated (Fig. 2, c).

Some PMNs were seen in the earliest period (24 to 72 hr) in the corneal stroma, particularly at the margins of the injected material (Fig. 2, a). These PMNs appeared after most injections, including control cells, latex spheres, and stimulated cells. Most were presumably derived from the tear film and injection tract; some could be seen entrapped in the corneal epithelium. Electron microscopy showed the reactive cells in the vicinity of the injection sites to the fibroblasts and macrophages. After several weeks, most of the injection sites had been cleared of the debris, leaving behind an area of irregular basophilic stroma and fibroblastic cells.

**Limbus.** At 3 days, the limbus and adjacent stroma of corneas injected with stimulated lymphocytes showed mild polymorphonuclear infiltration. Engorged vascular channels were also noted. In the 3 to 5 day period, an increase in perivasculocellularularity at the limbus was observed. Neovascular sprouts extending into the superficial corneal stroma were accompanied by perivasculocellular cells. At 7 days, the cellular activity at the limbus was dominated by mononuclear cells (Fig. 3, a). Electron microscopy showed the limbal infiltrates to consist primarily of lymphocytes and macrophages (Fig. 3, b). The limbus and intervening stroma of corneas injected with control cells generally showed little cellular or neovascular response. In corneas that vascularized after injections of control cells, the histologic appearance was comparable to that of corneas injected with stimulated cells.

**Discussion**

Corneal neovascularization was induced by the intrastromal injection of allogeneic lymphocytes in rabbits, an effect significantly enhanced when the lymphocytes had been stimulated in vitro by Con A. Although these experiments confirm other reports of the same phenomenon, our study demonstrates that the effect may be more host-dependent than originally appreciated. Although the angiogenic response may result from direct activation of endothelial cells by lymphocyte mediators, such as prostaglandins, the present observations show the angiogenic response to occur in the context of an inflammatory host vs. graft reaction.

The vascularization promoted by Con A–treated cells in our experiments is very likely based on enhanced immunologic recognition of the injected cells by the host. This was indicated by (1) the absence of corneal neovascularization after injections of autogeneic stimulated cells even when they were placed immediately adjacent to the limbus; (2) variability among nonlittermate rabbits to injections of Con A–stimulated lymphocytes from the same culture; and (3) the striking round cell reaction at the limbus suggestive of such a host-immune response.

Because unstimulated allogeneic cells did not effectively provoke such cellular and neovascular reactions, Con A treatment in some way amplified the host response. This was presumably not due to inflammatory reactions to the Con A itself, because the autogeneic and the killed cells did not evoke angiogenic reactions, although they were treated with Con A and washed in the same fashion as the stimulated allogeneic cells. The enhanced host reaction, instead, may be affected by increased expression of histocompatibility antigens known to occur after mitogen-induced blast transformation or because of their enhanced lymphokine pro-
Fig. 3. Limbal response to injection of stimulated lymphocytes. a, At 7 days, the limbus is dominated by mononuclear cells and vessels extend into the stroma. (×298.) **Inset,** Radioautograph shows uptake of $^{3}H$-thymidine by vascular endothelium (E), and perivascular round cells (P). (×566.) b, Limbal reaction is composed primarily of lymphocytes (L) and monocytes (M). (×6217.)
duction. Con A, primarily a T lymphocyte mitogen, induces blast transformation with enhanced production of inflammatory mediators such as lymphokines and prostaglandins. Prendergast et al. have recently found that an extract from the seastar that possesses lymphokine-like activity produces corneal neovascularization accompanied by a marked inflammatory response. Although supernatants from stimulated cultures failed to induce neovascularization when injected directly into the corneal stroma in our system, BenEzra and Pliskin have successfully produced neovascularization by implants of supernatants in slow-release polymers.

The importance of such sustained stimuli in promoting neovascular growth has been noted by others. The failure of stimulated killed cells to induce the response indicated that sustained production of angiogenic substances by viable cells was necessary. In addition, prolonged intrastromal viability of injected cells was clearly important. Stimulated cells were observed to take up 3H-thymidine after 2 to 3 days in the cornea, and electron microscopy showed these cells to be viable for significantly longer periods of time than their nonstimulated counterparts. The importance of a persistent stimulus was further suggested by the coincidence of neovascular involution with necrosis of the injected cells.

The striking mononuclear responses that predominated after injections of stimulated allogeneic cells were different from the PMN infiltrates that Klintworth and associates have identified in other models of experimentally induced corneal neovascularization. The role of the PMN may be facilitative, since neovascularization has been induced even in animals depleted or devoid of PMNs. PMN infiltrates seen with control injections did not seem to be sufficient to generate neovascularization. The presence of some PMNs after injections of stimulated allogeneic cells, however, cannot be ignored because they could provide an early preconditioning stimulus to which the subsequent mononuclear reaction is added. This idea was supported by preliminary experiments (data not shown) utilizing the technique of Goldbaum et al. to deplete rabbits of circulating PMNs with mechlorethamine. Such treatments did abolish cellular and neovascular responses to injections of stimulated allogeneic cells, which suggests an early intermediary role for the PMN.

The predominance of the mononuclear reaction seen at the limbus of corneas injected with stimulated cells between the fifth and seventh days evidently provided the additional stimulus necessary to provoke neovascularization under the conditions of our study. The lymphocytes and macrophages that participated in this reaction have themselves been reported to have angiogenic capabilities. This additional inflammatory stimulus or some effect more directly related to the heightened host recognition of Con A–stimulated allogeneic cells may promote the neovascular response.

The possibility that activated lymphocytes can facilitate the angiogenic response in a manner dependent on antigenic recognition is consistent with recent reports demonstrating immunologic modulation of some forms of neovascularization. Facilitation of angiogenesis by antigenic recognition may be important in several respects. Although the precise relationship between allograft rejection and vascularization after keratoplasty is not yet clear, it is possible that such neovascularization is promoted by similar actions of lymphocytes specifically sensitized against the graft. In angiogenesis induced by implantation of tumor cells into the cornea, the host-immune reaction to the heterogenic implants is an important consideration, as originally suggested by Sidky and Auerbach and more recently by Pliskin, especially in light of the finding that TAF can be antigenic. The apparent importance of host recognition in the present experiments likewise suggests that antigenicity of corneal implants may play a significant role in their ability to evoke neovascular reactions.

We thank Mrs. J. Aras for the preparation of histologic material, Dr. T. Lint and the Department of Immunology for their assistance and the use of their facilities, and Ms. Jo Schmidt for typing the manuscript.
REFERENCES


