Anterior chamber injection of donor rabbit lymphocytes sensitized in vitro to recipient alloantigens leads to the development of small focal areas of endothelial cell destruction (pocks) on the recipient cornea. Damage may be observed through a specular microscope as early as 2 days after injection of sensitized lymphocytes. Recipients of unsensitized allogeneic or sensitized autologous lymphocytes demonstrate little or no endothelial damage and no pock formation. Flat endothelial preparations reveal focal destruction of the endothelium with multiple foci, many infiltrated and surrounded by mononuclear cells. This model provides controlled sensitization to a variety of histocompatibility and corneal antigens that may be responsible for initiation of graft rejection. Invest Ophthalmol Vis Sci 26:116-121, 1985

Maintenance of a functionally intact donor endothelium is of critical importance in keratoplasty. Allograft rejection may produce severe damage to this layer of the cornea, which results in hydration and clouding of the stroma and loss of vision. For these reasons, an understanding of the interactions between lymphoid and endothelial cells is of utmost importance.

A model of endothelial allograft rejection was reported previously in which pocks of focal destruction of corneal endothelium were produced by lymphocytes from a donor sensitized to the recipient by prior skin grafting. This reaction was shown to be allospecific, since it was demonstrated that only appropriately histoincompatible corneas suffered destruction. The model is invaluable for assaying interactions between lymphoid and corneal endothelial cells that is of utmost importance.

Materials and Methods. Animals: All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research. A total of 32 outbred New Zealand white rabbits weighing 3-4 kg were used (Bunnyville; Littlestown, PA).

In vitro sensitization: Pairs of rabbits were anesthetized with an intramuscular injection of 44 mg/kg ketamine HCl (Ketalar, Parke-Davis) followed by a subcutaneous injection of 0.04 mg/kg atropine sulfate and 35 to 50 ml of blood was collected into a heparinized syringe by cardiac puncture. Equal amounts of whole blood and 3% pigskin gelatin (Sigma, Type II) in phosphate-buffered saline (PBS) were mixed and incubated at 37°C for 15 min to hasten erythrocyte settling. Peripheral blood mononuclear leucocytes (PBML) were obtained by underlaying the cell suspension above the erythrocytes on a Ficoll-Hypaque density gradient as described previously. PBML were harvested from the interface of the gradient, washed twice in PBS, counted and adjusted to 2 X 10^6/ml in RPMI 1640 (Gibco) supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS).

Culture conditions: Reciprocal one-way-mixed lymphocyte cultures (MLCs) were set up in 25-cm²
flasks (Corning) using a 5-ml aliquot of PBML obtained from one rabbit as responder cells and a 5-ml aliquot of irradiated (3,000 rad) PBML obtained from a second rabbit as stimulator cells, in a total volume of 10 ml complete RPMI. Flasks were incubated in an upright position for 2, 4, or 6 days at 37°C in 5% CO₂. Effector (sensitized) cells were harvested, washed, counted, and adjusted to 10 x 10⁶/ml in PBS. Cell viability as determined by trypan blue exclusion was generally 50%.

**Anterior chamber injection:** Under general anesthesia, 0.1 ml of cell suspension (1 x 10⁶ sensitized PBML) was injected into the right anterior chamber (AC) of each rabbit as previously described.¹ Control left eyes were injected with an equal number of unsensitized allogeneic PBML (incubated 4 days but without stimulator cells) or sensitized autologous (lymphocytes that have been sensitized to alloantigens of a second rabbit and that are autologous to the rabbit whose anterior chamber is being injected) PBML.

**Clinical observations:** Eyes were examined daily by hand-light, slit-lamp, and specular microscope to follow the appearance and development of inflammation, keratic precipitates (KP), endothelial damage, and pock formation. Animals were killed on day 1, 2, 4, or 6 for histologic studies.

**Histology:** Eyes were enucleated and the corneas removed for the preparation of flatmounts of corneal endothelium according to a technique previously described.⁴ Endothelia first were silver stained and then counterstained with Xanthene and Methylene Blue (Diff-Quik, Scientific Products) for visualization of lymphoid cells.

**Results. Clinical response:** Within 24 hr after injection of 1 x 10⁶ in vitro 4-day sensitized allogeneic PBML revealed the presence of scattered inflammatory (lymphoid) cells over the endothelium (Fig. 2A). The endothelium itself appeared undamaged and retained its normal and regular pattern. More cells were attached to what had been the lower portion of the endothelium than to the upper area. By day 2, these eyes demonstrated clusters of lymphoid cells on the endothelium, and these clusters surrounded areas of focal destruction of the endothelial layer (Fig. 2B). The endothelium surrounding the foci was disrupted and displayed an irregular pattern. By day 6, eyes that had received sensitized allogeneic cells displayed a decrease in the size of pocks and mitotic figures observed on the endothelium suggested a healing process (Fig. 2C).

Endothelial flatmounts prepared from control left eyes that had received either 1 x 10⁶ in vitro sensitized autologous PBML or 1 x 10⁶ unsensitized allogeneic PBML revealed the presence of scattered inflammatory cells at 24 hr after injection (Fig. 3a). By day 2, lymphoid cells were still present, but clusters of lymphoid cells were not observed, and the entire endothelium retained a normal, undisturbed pattern (Fig. 3b). On day 6, no lymphoid cells could be observed in flatmounts prepared from these eyes (Fig. 3c).

**Discussion.** We have demonstrated that peripheral blood mononuclear leucocytes obtained from one rabbit and sensitized in vitro to the alloantigens of a second recipient rabbit are capable of producing endothelial destruction when injected into the anterior chamber of the recipient animal. The endothelial damage appears to be identical to damage observed when sensitization is achieved through skin grafting, as previously reported.¹ Some minor differences, however, exist in the kinetics of the pock assay between the two sensitization protocols. In the skin allograft model, the anterior chamber inflammatory reaction appeared to peak between 5 and 7 days after inoculation of sensitized lymph node cells.¹ In the current in vitro model, peak inflammation and pock formation was noted by day 2. This discrepancy in the kinetics of endothelial destruction may be due to the number of specifically sensitized cells injected.

This hypothesis is supported by a recent study in which lymph node cells obtained from the draining lymph node of a skin graft recipient were restimulated ("supersensitized") in vitro by a second exposure to
mitomycin C-treated PBML from the original skin donor for 3 days prior to AC injection (Khodadoust, personal communication). Under those conditions, endothelial pocks were observed within 2–3 days after injection.

In vitro sensitization of alloreactive PBML that have in vivo activity was reported several years ago. In those experiments, in vitro sensitization of mouse thymocytes to alloantigens produced effector lymphoid cells which were capable of inducing rejection of allogeneic skin grafts after injection into T-cell depleted, but not lethally irradiated mice. This suggests that in vitro sensitized cells did not directly attack the graft alone, but more likely secreted lymphokines that recruited the recipient's own effector population. In the current model, the origin of the cell causing endothelial pocks is not known, although it has been demonstrated previously that sensitized T-cells are involved.
Fig. 2. Micrograph depicting flat endothelial preparation after injection of $1 \times 10^6$ in vitro sensitized allogeneic lymphocytes into the anterior chamber. A, 24 hr after injection scattered lymphoid cells are observed; B, 2 days after injection clusters of lymphoid cells forming holes in the endothelium, and the normal pattern of endothelial cells is disrupted around these holes; C, 6 days after injection pocks are still evident although few lymphoid cells remain; numerous mitotic figures suggest endothelial healing (magnification $\times 40$).
Fig. 3. Micrograph depicting flat endothelial preparation after injection of $1 \times 10^6$ in vitro sensitized autologous lymphocytes into the anterior chamber. A, 24 hr after injection scattered lymphoid cells are observed; B, 2 days after injection the number of lymphoid cells has decreased—no holes are apparent and the endothelium retains its regular pattern; C, 6 days after injection the control eye is completely normal with no evidence of pocks or lymphoid cells (magnification $\times 40$).
absolutely required to initiate pock formation. Studies currently in progress will examine the role of donor and recipient effector cells.

Our findings demonstrate that sensitization to lymphocyte antigens may lead to allograft rejection against donor corneal endothelial cells, and this has important implications for human graft recipients who have received previous blood transfusions. This new, simplified model of corneal allograft rejection will permit controlled sensitization to a variety of corneal and histocompatibility antigens, which may be responsible for initiation of graft rejection. The model is ideal for studying the sensitization of isolated lymphocyte populations, and their interactions with cells of the corneal endothelium in vivo and allows for experimental manipulations during the sensitization phase, which may lead eventually to a means of completely abrogating the corneal allograft reaction.

Key words: in vitro sensitization, corneal rejection, endothelial cell pock formation

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References


HSV-1 Quantitation from Rabbit Neural Tissues after Epinephrine-Induced Reactivation

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Epinephrine iontophoresis into the eye can induce ocular herpes simplex virus type-1 (HSV-1) shedding with a high frequency from latently infected rabbits. The present study was designed to quantify and quantify infectious HSV-1 from neural tissues of latently infected rabbits after ocular epinephrine iontophoresis. Epinephrine iontophoresis was performed daily for 3 consecutive days on selected days during 220–227 days postinoculation. The induced ocular shedding was detected in the tear film with a frequency of 83% (10/12) within 72 hr after the initial iontophoresis. The rabbits were killed 24 hr after the last iontophoretic treatment, and the corneas and neural tissues were homogenized immediately. The cell-free supernatants were inoculated on primary rabbit kidney cell monolayers for qualitative assays of infectious virus and later titrated on CV-1 monolayers. The frequencies of the recovery of infectious HSV-1 from the cell-free homogenates were 0% of the corneas (0/12), 83% (10/12) from the superior cervical ganglion (SCG), 100% (12/12) from the trigeminal ganglion (TG), 42% (5/12) from the ophthalmic branch of the trigeminal nerve (TN), 8% (1/12) from the root entry zone of the trigeminal nerve into the brain-stem (REZ), and 0% (0/12) from the cerebellum. The authors conclude that epinephrine iontophoresis can reactivate the latent HSV-1 in neural tissues and infectious virus can be quantified from the cell-free homogenates. To the best of our knowledge, this is the first report to quantify HSV-1 with a high frequency from neural tissues following induced reactivation. Invest Ophthalmol Vis Sci 26:121–125, 1985

Investigations to elucidate the mechanisms of HSV latency, reactivation, and recurrences require a reproducible and reliable animal model. We have demonstrated that epinephrine iontophoresis to the cornea induced ocular HSV-1 shedding reliably from latently infected rabbits.1–3 In ocular HSV infections, two methods are available to detect reactivation; one is to determine ocular shedding,1–6 and another is to determine the presence of infectious virus in the neural tissues.2–7 During latency, infectious virus cannot be detected in cell-free homogenates of ganglia but can be detected by explant co-cultivation. In our previous study, infectious HSV-1 was found only in cell-free homogenates of neural tissues after an in vitro 18–24 hr amplification.1 We failed to detect infectious virus from direct (no amplification) homogenization of neural tissues in latently infected rabbits that were killed 4 days after the last epinephrine iontophoresis. These results suggest that an earlier killing might provide more information about an