until results of additional, carefully controlled prospective studies become available.11

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REFERENCES

Instillation of Steroids in the Eye

Its Effect on Lymphocytes in Regional Lymph Nodes and in Peripheral Blood

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SUMMARY
The blastogenic response to mitogens by lymphocytes after topical instillation of steroids was studied in rabbits. The effect on lymphocytes from the local draining lymph nodes and peripheral blood was assessed. There was no effect on the lymphocytes of local draining lymph nodes; however, a decrease in phytohaemagglutinin response occurred in peripheral blood lymphocytes.


Corneal graft rejection is treated clinically by both systemic and topical corticosteroid therapy. Local instillation affects lymphocytes that assemble in the eye as part of the rejection process.1 The purpose of this study was to determine the effect of topically administered steroids on the lymphocytes in the local draining lymph nodes and on peripheral blood lymphocytes. These lymphocytes were assessed for their ability to undergo blastogenesis in the presence of various mitogens.

MATERIAL AND METHODS
Adult New Zealand White rabbits were divided into two groups. The first consisted of 10 rabbits that received no treatment (control group). The second consisted of 6 rabbits treated with two drops of a solution of betamethasone sodium phosphate 1 mg/ml (Betnesol; Glaxo) placed in the right eye five times daily at 2-hourly intervals for a period of 2 weeks (steroid therapy group). Each animal received a total of 4 mg betamethasone sodium phosphate. The lymph nodes draining the right and left eyes were removed under pentobarbitone anaesthesia. The node was located by making an incision parallel to the longitudinal axis of the body starting 1.5 cm behind the corner of the eye and extending to just below the lower cartilage of the ear. The lymph nodes were placed in Hanks's balanced salt solution (HBSS) immediately and excess connective tissue was removed and the cells were teased out of the node into fresh medium. A single-cell suspension was made by pipetting the suspension up and down and then allowing the clumps and large debris to settle. These cells were then washed three times in HBSS by centrifugation at 200 g for 10 minutes.
The peripheral blood lymphocytes were obtained by withdrawing 20 ml of blood by cardiac puncture into heparinized syringes (0.2 ml of sodium heparin 1 000 U/ml without preservatives). The blood was then diluted 1 : 2 with HBBS and layered onto a Ficoll-Hypaque gradient and centrifuged at 1 200 g at the interface at 18°C for 30 minutes. The lymphocytes, which collected at the interface, were removed and washed four times in HBSS by centrifugation at 200 g for 10 minutes, to remove any Ficoll-Hypaque and platelets. The lymphocytes were examined for their ability to respond to blast transformation by three different mitogens: (a) purified phytohaemagglutinin (PHA) (Wellcome Reagents) 20 μl used per sample of a 50 mg/ml solution in normal saline; (b) concanavalin A (con A) (Pharmacia Fine Chemicals), 20 μl per sample of a 1 mg/ml solution in normal saline; (c) pokeweed mitogen (PWM) (Barker and Sarnes, Grand Island Biological Co., New York), 20 μl per sample of a 1/5 dilution in normal saline (0.9%).

The tissue culture medium throughout was RPMI 1640 (Flow Laboratories) supplemented with 20% normal rabbit serum which had been heat-inactivated at 56°C for 30 minutes, 500 mM glutamine, 50 mg/ml gentamicin and 20 mM Hepes. The final pH was adjusted to 7.2 with 1N sodium hydroxide.

The assay was performed in quadruplicate. Aliquots of 130 μl culture medium containing 1.5 × 10⁸ lymphocytes were used per well of a U-shaped Cooke microtitre tissue culture plate; 20 μl of mitogen was added to this and the plate was sealed and placed in a 37°C incubator. Lymphocytes without mitogens added were used as controls. Lymphocytes stimulated by PHA and con A and the controls were cultured for 48 hours; those stimulated by PWM were cultured for 4 days. Cultures were then pulsed with 0.2 μCi 6-H-thymidine (Code TRK 296, Radiochemical Centre, Amersham, England) for a further 16-18 hours. Cells were then harvested on a 12-well water-wash Skatron Harvester (Flow Laboratories) using Skatron harvesting paper. After air-drying the filter discs were placed in capped vials with 10 ml scintillation fluid (5 g PPO + 0.1 g Bis MSB/1 toluene) and each sample counted for 10 minutes on a Packard Tri-Carb counter. The results were expressed as a stimulation index — ratio of control to test (control taken as 1), i.e. 1 : x where x = test/control. The results were expressed as logs because the data were in the form of a ratio and therefore tended to have a skew distribution.

RESULTS

The results are shown in Tables I - III.

In Table I the effect on the lymphocytes from the draining lymph node of the right eye (steroid-treated) has been compared with that of the untreated left eye. The results were tested by the Wilcoxon test and showed no significant difference between the two sides. Similarly there was, by the Wilcoxon test, no significant difference in the comparison of the lymphocytes in the lymph nodes of the right and left eyes of the untreated control group of animals (Table II). As there was no difference in the transformation of the lymphocytes of the right and left eyes in the steroid-treated and control groups, the results in each of these animals were averaged and compared. The total results were compared by the Mann-Whitney U test. There was no statistically significant difference between the two groups.

The results of the peripheral blood lymphocyte transformations may be seen in Table III. These were analysed by the Mann-Whitney U test; the results with PHA stimulation showed a significant difference (P = 0.05) while the other mitogens resulted in no significant difference.

**DISCUSSION**

The role that the local draining lymph node plays in corneal graft rejection is not fully elucidated. During the rejection process in other parts of the body it is assumed that histocompatibility antigens travel by way of lymphatic channels to the regional lymph nodes. The cornea lacks such channels, but Polack suggests that this function may be carried out by interfibrillar and interlamellar spaces. In fact, he noted that with corneal heterografts changes in regional lymphatic tissue occurred before the cornea became invaded by round cells.

Corneal graft rejection can be partially reversed by topical instillation of steroids. According to Brent, in patients with a kidney graft one is treating the patient's entire lymphatic system, while in the cornea, with topical steroids, one is treating whatever lymphocytes happen to be in the graft. He poses the question whether the steroid reaches the regional lymph nodes. The results in this study show that in a rabbit model, after the instillation of steroids, there is no effect on the lymphocytes of the local draining lymph nodes as judged by their ability to undergo blastogenesis in the presence of various mitogens. This
may be due to a failure of the steroids to reach the lymph nodes. We have instilled trypan blue into the eyes of rabbits and observed its presence macroscopically and microscopically in the local draining lymph nodes. Whether steroids follow a similar pathway after local instillation is not known. A review of the literature has failed to reveal any information on the effect of topical application of steroids on local lymph nodes. It is of significance that the instillation of topical steroids resulted in a decrease in PHA-stimulated blastogenesis in the peripheral blood lymphocytes. A similar reduction in T-lymphocyte function caused by corticosteroid administration, as judged by PHA response, has been noted in man by Cooper et al. They administered the steroids orally daily for a 3-week period to patients requiring corticosteroid therapy and noted a decrease in PHA-stimulated blastogenesis in peripheral blood lymphocytes. The dose of steroids received by these patients was equivalent on a weight-for-weight basis to that which was given to the rabbits in our study. It would therefore appear that the steroids are absorbed, probably through the aqueous into the circulation, with resultant effect on the peripheral blood lymphocytes. Whether this effect plays a part in the control of the corneal graft rejection process is not clear. The decrease in PHA stimulation in the presence of corticosteroid administration would indicate a reduction in T-lymphocyte function, i.e. the lymphocyte that is the effector cell in cell-mediated immunity and graft rejection.

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REFERENCES