Induction of delayed-type sensitivity-like reactions in the eye by the injection of lymphokines*

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Biologically quiescent small lymphocytes from guinea pigs were placed in contact with a variety of stimuli in vitro and were transformed into active cells as demonstrated by changes in their morphology, production of DNA, and cell division. In association with these biologic events, the stimulated lymphocytes have been shown to release soluble products (“lymphokines”) in vitro which exhibit the capacity to kill cells in tissue culture (except lymphocytes), inhibit the migration of macrophages, and produce skin and corneal reactions which are histologically identical to delayed-type sensitivity reactions. In the present investigations, intralamellar corneal and intraocular injections of lymphokines were studied. Corneal injections of lymphokines produced by mixed cultures of allogenic lymphocytes result in inflammatory reactions that closely resembled tuberculin-induced corneal reactions in BCG-immunized guinea pigs. Intraocular injections of lymphokines produced inflammatory exudates which consisted primarily of lymphocytes and macrophages. These studies indicate that lymphokines produced by the interaction of sensitized lymphocytes with antigen are capable of eliciting ocular inflammatory reactions which closely resemble delayed-type hypersensitivity reactions.

Key words: cornea, ocular immunology, uveitis, lymphocytes, delayed-type hypersensitivity, mediators of cellular immunity, inflammation, lymphokines.

In previous experiments the possible key role of soluble products (lymphokines) released by antigen-stimulated lymphocytes in the production of corneal inflammation was demonstrated. In those studies, lymphocytes from BCG-immunized guinea pigs were cultured in the presence of tuberculin. The resultant culture supernatant was capable of producing inflammatory reactions when injected into either skin or cornea of unimmunized animals which were histologically similar to tuberculin reactions in immunized animals.
These studies suggested that delayed-type sensitivity reactions are capable of producing corneal inflammation.

The present studies demonstrate that lymphokines produced by mixed cultures of allogenic lymphocytes are also capable of producing corneal inflammation which is identical to that previously described. In addition, further evidence of the importance of delayed-type sensitivity reactions in the production of ocular inflammation is achieved by intraocular injections of lymphokines. Injection of lymphokines into either the anterior chamber or vitreous produced anterior-segment inflammation.

Materials and methods

Animals. Adult inbred Strain-13 and colony Chase guinea pigs were used throughout the experiments.

Sensitization. Strain-13 guinea pigs were sensitized with 2.4 mg. of heat-killed organisms of the BCG strain of Mycobacterium tuberculosis suspended in 0.6 ml of Bayol F. One-tenth milliliter of this suspension was injected subcutaneously into each of six sites on the back of each animal. One month later a few representative animals of each group were given tuberculin skin tests to assure an adequate general level of sensitivity. Only animals of groups that exhibited necrotic skin test reactions to 10 µg purified protein derivative (PPD) were used as lymphocyte donors. Normal, unsensitized Strain-13 and Chase guinea pigs were used as lymphocyte donors.

Corneal tuberculin tests. Prior to injection, two drops of 0.5 per cent proparacaine hydrochloride (ophthalmic solution) were dropped on the cornea and the animal was lightly anesthetized with ether. Treated tuberculin (TT) of Middlebrook (2.5 µg) which is essentially carbohydrate-free* in a volume of 0.03 ml. was injected intralamellarly into the cornea with a 30-gauge needle attached to a 0.25 ml. tuberculin syringe.° In guinea pig studies, 10 µg PPD per milliliter was added to the medium. The cultures were incubated for 24 to 48 hours at 36.5° C. in a humid atmosphere containing 5 per cent CO₂. The test sites were examined at frequent intervals after injection. The reactions were graded on the basis of a 0 to 4+ scale employed previously by Smith and Weiser. Reactions graded 1+ showed slight clouding; those graded 2+ showed moderate clouding; those graded 3+ showed severe clouding with the outlines of the pupil barely visible; and those graded 4+ showed intense clouding with complete obliteration of the outlines of the pupil.

Production of lymphokine preparation (LP).* Experiments indicated that the viability of cultured cells could be maintained for 48 hours in the absence of serum when placed in MEM supplemented with L-glutamine, nonessential amino acids,† and 100 U. per milliliter of penicillin and streptomycin 100 µg per milliliter.

For the tuberculin-produced LP, the following culture system was utilized. Approximately 1 x 10⁶ viable lymphocytes were employed and 25 µg PPD per milliliter was added to the medium. Control supernatants were prepared by incubating the lymphocytes in the absence of PPD; PPD was finally added to the filtered supernatants. An additional control consisted of fresh MEM plus PPD.

In the mixed lymphocyte cultures, 1.2 x 10⁶ viable cells per milliliter (0.6 x 10⁶ cells from each animal) were employed. Control samples consisted of 1.2 x 10⁶ cells per milliliter from a single animal.

The cultures were incubated for 24 to 48 hours at 36.5° C. in a humid atmosphere containing 5 per cent CO₂. At the completion of the incubation

*Lyphokines" is the general term for soluble factors released in vitro in models of delayed-type sensitivity reactions. In contrast, lymphokine preparation (LP) is the term we use to identify the crude material that is the subject of these in vivo studies.

†The nonessential amino acid solution was obtained from Grand Island Biological Company, Grand Island, N. Y.
Table I. Average of clinical grading of the evolution of corneal reactions

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<td>BCC-sensitized GP (n = 10)</td>
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<td>TT 2.5 μg*</td>
<td>1+</td>
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<td>Saline control</td>
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<td>Normal GP (n = 16)</td>
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<td>LP 30 μg†</td>
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*Treated tuberculin of Middlebrook.†Lymphokine preparation produced in mixed cultures of allogenic lymphocytes.

Intracorneal injections of LP. By utilizing the culture methods detailed above, it has been possible to consistently produce LP. Although the protein content and inflammatory capabilities of different pools of material varied, supernatants from antigen-stimulated cells from sensitive animals or supernatants from mixed lymphocyte cultures were always much more cytotoxic than control supernatants. The ability of LP to destroy cultured mouse L-cell monolayers has been reported previously.1,6

The sequential responses observed clinically in guinea pigs' corneas injected with LP from mixed cultures of allogenic lymphocytes is summarized in Table I. The onset of significant corneal reactions was noticeable in all cases by six hours. The LP reactions reached their peak of intensity 11 to 18 hours after injection. In contrast, the corneal tuberculin reactions reached maximal intensity at 18 to 24 hours by which time the LP reactions were beginning to significantly decrease in intensity. The corneal tuberculin reactions began to recede after 24 hours when 2.5 μg TT were used; those induced with larger doses of TT persisted for longer periods. In all instances guinea pigs injected with LP received a higher rating at 11 hours than at later times. In contrast, animals injected with TT always had higher scores at the later time intervals. The control supernatants in all cases gave no clinical reaction after the initial corneal clouding coincident with injection. These sequential responses were similar to those observed when PPD-induced LP was injected into either skin or cornea in a previous investigation.1

Histologic sections of corneas from guinea pigs killed at various intervals were studied. Corneas injected with control materials showed no accumulation of cells in the limbal region (Fig. 1). After the injection of LP into the corneas of normal
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guinea pigs, an accumulation of cells, predominantly mononuclear, began at the limbus in approximately six hours. The limbal infiltrate continued to increase through 18 hours (Fig. 2), and by 48 hours had almost completely disappeared. In contrast, the corneal reactions induced by TT in BCG-sensitized animals showed a similar mononuclear cell limbal infiltrate at six hours which persisted for at least 48 hours (Fig. 3). Although the intensity of the infiltrate often varied between the two systems, the predominance of mononuclear cells was evident in each case and the histology was, for all practical purposes, identical.

The migration of cells from the limbal region into the corneal stroma coincided with the onset of clinically evident corneal clouding. The stromal infiltrate consisted almost exclusively of polymorphonuclear leukocytes tightly packed between stromal lamellae irrespective of whether LP or TT was injected (Fig. 4). Abundant cellular infiltrates persisted after corneal clouding had diminished. Corneas injected with control preparations showed no clouding and no cellular stromal infiltrate (Fig. 5). The histology of the reactions produced by LP from mixed cultures of allogenic lymphocytes was essentially identical to that produced by PPD-induced LP.³

Intraocular injections of LP. All eyes injected with LP developed limbal hyperemia by 6 hours; hyperemia was still present in animals killed at 18 hours. There were no instances of corneal clouding after intraocular injections of LP. The eyes injected with control material remained normal and the corneas clear. Slit lamp biomicroscopy was not employed in these experiments.

Histologic examination of eyes after anterior chamber injections of control material failed to show any evidence of an inflammatory reaction (Fig. 6). In contrast, eyes injected with LP showed intense cellular reactions with inflammatory cells in the limbus, iris, ciliary body, and anterior chamber (Figs. 7 and 8). In some cases, the anterior chamber also contained a proteinaceous exudate.
Intravitreal injections of LP also elicited inflammatory reactions. Inflammatory cells were found in the iris, ciliary body, and anterior chamber as well as in the vitreous (Fig. 9). Intravitreal injections of control material failed to produce any inflammatory response (Fig. 10). The inflammatory cell population was composed predominantly of small and large mononuclear cells and a few polymorphonuclear leukocytes.

Inflammatory foci did not develop in the choroid of the animals after the injection of LP and no accumulation of inflammatory cells occurred at the optic disc.

Discussion

The in vitro production and release of substances by immunologically stimulated lymphocytes and macrophages from many species, including guinea pigs and man, has been established in many laboratories.\(^1,5,9-14\) The present study, as well as another companion investigation\(^1\) were undertaken to investigate the in vivo capabilities of these substances and to correlate them with delayed-type sensitivity reactions.

Clinical investigators are beginning to appreciate that certain systemic immunologic deficiencies, previously of unknown etiology, may rest on incompetent lymphocytes which lack the capacity to produce lymphokines. The etiologic role that these substances may play in ophthalmic disease is speculative.

The production of "lymphokines" begins 2 to 3 hours after exposure to either specific or nonspecific mitogens and requires energy and protein synthesis. It precedes and appears to be independent of DNA synthesis.\(^10,11\) Normal lymphocytes typically show 3 to 4 small, discrete lysosomes in a perinuclear position. In stimulated lymphocytes an increase in the number and size of lysosomes is noted as early as 12 hours and appears to precede mitosis.\(^12,13,14\) Since LP is present in the medium in significant quantities at three hours, it is unlikely that lysosomal enzymes are responsible for the cytotoxic and inflammagenic capabilities of LP.

The histology of the LP corneal reactions in normal guinea pigs was constant.
Fig. 4. Central cornea of unimmunized guinea pig 12 hours after the injection of lymphokine preparation. (Hematoxylin and eosin, magnification ×125.)

Fig. 5. Central cornea of unimmunized guinea pig 12 hours after the injection of control lymphocyte preparation. (Hematoxylin and eosin, magnification ×125.)
Fig. 6. Anterior segment of guinea pig eye 6 hours after anterior chamber injection of control lymphocyte preparation. (Hematoxylin and eosin, magnification x200.)

Fig. 7. Anterior segment of guinea pig eye 6 hours after anterior chamber injection of lymphokine preparation. (Hematoxylin and eosin, magnification x250.)
Fig. 8. Anterior segment of guinea pig eye 18 hours after anterior chamber injection of lymphokine preparation. (Hematoxylin and eosin, magnification x250.)

Fig. 9. Ciliary body, lens, and vitreous of guinea pig eye 18 hours after intravitreal injection of lymphokine preparation. (Hematoxylin and eosin, magnification x250.)
and closely resembled the tuberculin corneal reactions in BCG-sensitized animals. It is well-known that the cells which migrate into the stroma in corneal tuberculin reactions are largely polymorphonuclear leukocytes (PMN's). This same phenomenon occurred following the injection of LP suggesting that these substances have leukotactic activity, at least for PMN's. The failure of lymphocytes and macrophages to migrate into the corneal stroma may be due to their limited mobility or to the density of this structure. Ward, Remold, and David have demonstrated, in an in vitro system, that macrophage inhibitory factor (MIF) produced by stimulated lymphocytes is distinct from a factor which is leukotactic for mononuclear cells. In addition, they found a third factor which is chemotactic for neutrophils. Heise and Weiser have demonstrated that both crude and partially purified supernatants produced in a tuberculin system analogous to that employed in the present experiments possesses both LP and MIF activity. The limbal infiltrate is composed primarily of mononuclear cells, and this is probably where the initial interaction between antigen and specific antigen-sensitive cells takes place. Presumably, this interaction allows the subsequent inflammatory response to occur. The lack of PMN's in the stroma in control reactions (and with TT in nonsensitized animals) clearly rules out a nonspecific response.

In a similar manner, the results with intracocular injections of LP are consistent with the concept that natural disease of the eye can be caused by delayed-type sensitivity reactions. The release of small quantities of lymphokines may lead to increased vascular permeability, attraction of leukocytes by chemotaxis, recruitment of nonsensitized lymphocytes to produce...
lymphokines, and immobilization, activation, and proliferation of macrophages. Furthermore, lymphocytes that remain in the eye after an initial reaction, or their progeny, or new infiltrating cells may be capable of reacting repeatedly with the same antigen or antigen-antibody complexes to produce recurrent inflammation.

REFERENCES


