
Several immunologic tests were used to compare the soluble antigens of cultured and normal bovine corneas. Results of fluorescent-antibody tests on normal corneas confirmed our earlier observation that the epithelium contained little serum protein, and that stroma and epithelium shared an antigen not found in bovine serum. Immunoelectrophoresis, immunodiffusion, and fluorescent-antibody tests performed on extracts prepared from normal and cultured corneas indicated that corneas absorbed serum proteins during the incubation period. We also noted that very little intact epithelium remained on corneas cultured in the presence of calf serum. The extract prepared from cultured corneas did not contain the strongly antigenic protein present in extracts of normal cornea or epithelium. The implications of these findings are discussed.

Our previous report described the soluble antigens extracted from bovine corneas. In addition to serum proteins, a protein was found in extracts of whole cornea and of corneal epithelium that was not found in bovine serum. This strongly antigenic protein was similar in distribution and electrrophoretic mobility to a protein described earlier by Holt and Kinoshita and to a protein described by Whiteside, Hamada, and Manski.

The present experiments were designed to determine the antigenic nature of an extract prepared from cornea that had been cultured in vitro and to compare this extract with that prepared from normal corneas.

Materials and methods.

Culture methods. Bovine corneas, with a small rim of sclera, were cultured in 60 by 15 mm. plastic culture dishes containing Eagles' minimal essential medium (MEM), supplemented with 10 per cent heat-inactivated (56° C, 30 minutes) calf serum (CS), penicillin, and streptomycin. The cultures were maintained at 37° C in an atmosphere of 95 per cent air, 5 per cent CO₂. The medium was changed twice weekly. At the end of three weeks, the corneas that were free of bacterial or fungal contamination were frozen. Some corneas were cultured in MEM that contained 10 per cent normal heat-inactivated rabbit serum (NRS) instead of CS. We prepared a section of a representative cornea for routine histologic examination and a section for use in fluorescent-antibody studies.

Preparation of corneal extracts. Soluble proteins were extracted from normal and cultured corneas as described previously. We concentrated the material to a protein concentration of at least 20 mg per milliliter (Biuret). In one experiment, the culture medium was removed at each medium change and concentrated to approximately one-fourth its original volume.

Immunodiffusion and immunoelectrophoresis. These procedures were carried out as described previously. We tested the extracts from normal corneas (CA) and cultured corneas (CaC) against antiserum to bovine serum (anti-BS), anti-CA, anti-CaC, and anti-bovine gamma-globulin (BCG). These antisera were prepared in rabbits by an initial intravitreal injection of 0.1 ml of the material. A second intravitreal injection was given one week later. Booster intravenous injections were given at intervals in order to maintain the serum antibody titer at a high level. Hemolytic antibody titers of anti-CA and anti-CaC (against BCG-coated erythrocytes) were greater than 1:256.

The concentrated tissue culture medium was tested in immunoelectrophoresis studies against anti-CA and against anti-BS.

Fluorescent antibody (FA) studies. Sections of cultured and normal corneas were fixed in the solution described by Wolman and Behar and stained according to the technique described by Carver and Goldman. The sections were stained with the direct method, using fluorescein-conjugated goat anti-rabbit IgG (FL-RG) and fluorescein-conjugated goat anti-bovine IgG (FL-BG). Both antisera were obtained from Miles Laboratory, Elkhart, Ind. For indirect FA tests, we first reacted the sections with anti-BS, anti-CA, anti-BCG, or anti-EPI, and then with FL-RG.

Results.

Appearance of cultured corneas. Bovine corneas that had been cultured for three weeks were edematous and opaque. Their pink color indicated that they had absorbed the culture medium (containing phenol red) during the incubation period. Microscopic examination of stained sections (he-
matoxylin and eosin [H & E]) showed that the epithelium was almost entirely absent from the corneas that had been cultured in medium containing CS. We found some areas of intact epithelium in corneas cultured with NRS.

Immunological tests.

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS. Our previous studies showed that CA contained most of the major serum proteins but was not antigenically identical to bovine serum. A protein that migrated electrophoretically as an albumin appeared to be different from bovine serum albumin, because this protein failed to evoke antibodies that precipitated BSA. CA also contained a strongly antigenic protein that was not present in bovine serum. The protein could be demonstrated in immunodiffusion tests when CA was diluted 1:20. The present studies showed that the electrophoretic pattern of CaC was nearly identical to that of bovine serum (Fig. 1). When we used anti-CaC in the trough we obtained patterns very similar to those in Fig. 1. These observations suggested that serum proteins had been absorbed by the corneas during their incubation in MEM that contained CS.

Figs. 2 and 3 show the immunoelectrophoretic patterns of CA and CaC when they were tested with anti-CA or anti-EPI. It is evident that the strongly antigenic protein present in CA and EPI is absent from the extract prepared from cultured corneas. Three extracts prepared at different times produced identical patterns. We concentrated one CaC preparation to one-half its original volume and tested it against anti-EPI,
Fig. 3. Reaction of CaC, EPI, and CA with Anti-EPI. (1) CaC, (2) and (4) Anti-EPI, (3) EPI. (5) CA. Note absence of precipitation between anti-EPI and CaC.

anti-CaC, and anti-CA. There were no precipitin lines with anti-EPI, and only the serum proteins precipitated with anti-CA.

Tissue culture medium removed during the first two weeks and again immediately before harvesting the corneas was tested for the presence of the epithelial antigen. We were unable to demonstrate it in any of the concentrated medium samples. Bovine serum proteins were present, however.

FA TESTS. The stroma and endothelium of normal corneas showed a positive reaction in direct FA tests done with FL-BG. Stroma, epithelium, and endothelium of cultured corneas stained with FL-BC. Neither cornea stained with FL-RG. Specific fluorescence was observed in indirect tests performed on cultured and normal corneas when anti-BS, anti-BGG, or anti-CA were used in the first “layer.” The epithelium of normal corneas stained with anti-CA and anti-EPI (Fig. 4), but not with anti-BS or anti-BGG (Fig. 5). Anti-EPI stained the anterior third of the stroma of normal corneas (Fig. 4), but failed to stain cultured corneas.

TESTS WITH CORNEAS CULTURED IN MEDIUM THAT CONTAINED NRS. The preceding experiments indicated that a strong antigen present in CA and EPI was not present in CaC, or present...
in amounts too small to detect with our methods. They also suggested that cultured corneas absorbed bovine serum proteins from the culture medium. In order to substantiate the latter supposition, we cultured bovine corneas in medium that contained NRS, assuming that detection of rabbit serum proteins in the extract prepared from these corneas would provide definite proof of uptake of serum proteins.

The extract prepared from these corneas (CaR) contained bovine serum proteins as expected. The corneas had areas of intact epithelium, and the protein found in CA and EPI was present, although it was less concentrated (Fig. 6). We tested CaR in immunoelectrophoresis tests against unconjugated goat anti-rabbit IgG. These tests indicated that CaR contained substantial amounts of rabbit gamma-globulin. We were also able to show that anti-CaR (prepared in rabbits as described above) reacted with CA, EPI, and BS, but not with NRS.

FL-RG-stained corneas cultured in medium containing NRS, but did not stain normal corneas or corneas cultured in medium supplemented with CS. The fluorescence was eliminated when the sections were first treated with anti-rabbit serum that had been prepared in guinea pigs (GP-Anti-R). Indirect FA tests were positive when anti-BS, anti-CA, and anti-EPI were used in the middle "layer." The sections had first been treated with GP-anti-R to eliminate the positive reaction that would have resulted when FL-RG was used in the final step.

**Discussion.** These experiments, in addition to providing additional information on the antigenic nature of normal bovine corneas, have provided some information on the antigens of cultured bovine corneas. The results of our FA studies of normal corneas agree with the results of our previous immunochemical studies which showed that bovine corneas contained many of the bovine serum proteins. The failure of corneal epithelium to stain with anti-BGG substantiates our claim, and those of others, that corneal epithelium contains little or no serum proteins. Anti-EPI, which reacted primarily with the antigen common to CA and EPI, stained only the epithelium and the anterior third of the corneal stroma. Berger described an epithelial antigen but failed to find it in an extract prepared from the posterior two-thirds of bovine cornea, and Whiteside, Hamada, and Manski found a protein common to stroma and epithelium.

It has been claimed that corneas cultured for a period of time in vitro have longer survival times after transplantation than fresh corneas have, possibly because of altered antigenicity. The results of our experiments suggest that their antigenicity is indeed altered. Immunoelectrophoresis tests, immunodiffusion tests, and FA studies indicated that the extracts prepared from cultured corneas did not contain the strong antigen present in extracts prepared from normal corneas and from corneal epithelium. These results are consistent with our observation that little or no epithelium remained on the cultured corneas.
Although loss of epithelium may be primarily responsible for the loss of the antigen, the fact that stroma also contains the antigen must be considered. We did find the protein in an extract prepared from corneas from which the epithelium had been removed. We might postulate that stromal antigen diffused into the medium after the epithelium was lost. We did not find the antigen in the medium we tested, but the amount present at any one time could have been too small to detect, even in concentrated medium.

The observation that cultured corneas appear to have taken up serum proteins from the medium is perhaps more important. CaC very closely resembled bovine serum in its electrophoretic pattern and seemed to contain a greater concentration of serum proteins than we found in CA. Precipitin lines were heavier and more numerous. CaC contained the rabbit serum used in the culture medium, and sections from representative corneas stained with FL-RG. If cultured corneas are to be used in experimental or clinical transplantation situations, it would seem advisable to determine the possible immunologic effect of the additional proteins present. It would also be advisable to determine whether corneas cultured in medium containing serum from the potential host species would show increased survival time. Recent evidence indicates that corneas cultured in medium containing rabbit serum had a slower rejection time than we found in CA.

REFERENCES


Conjunctival goblet cell density in normal subjects and in dry eye syndromes.

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Serial sections prepared from biopsies of the deep tarsal portion of the inferior nasal conjunctival fornix in normal subjects and in patients with various dry eye syndromes were analyzed with respect to the goblet cell densities. When compared to normal subjects, individuals with keratitis sicca, Stevens-Johnson syndrome, ocular pemphigoid, and acute alkali burn all demonstrated progressively lower goblet cell densities per millimeter of epithelial surface. These disease entities can, therefore, be considered goblet cell-deficient syndromes.

The conjunctival goblet cell density may reflect the severity of local disease in the mucin-deficient dry eye syndromes. Diminution in number of goblet cells has been noted in ocular pemphigoid and avitaminosis A, while in keratoconjunctivitis sicca, Norn has described an apparent increase in mucus content of the tear film. Average goblet cell counts in normal subjects were first cited in 1910 by Virchow, who reported 10 goblet cells per millimeter on specimens from the upper orbital and tarsal areas, and 15 goblet cells per millimeter from the lower orbital and tarsal regions. From flat preparations of the entire conjunctiva, Keesing determined that the highest goblet cell counts, excluding the semilunar fold, were along the lower nasal oblique meridian. Examination of ten preparations, advancing centrifugally from the cornea along this meridian, resulted in an increasing goblet cell count reaching a peak along the tarsal conjunctiva.