Cell-surface receptors of normal, regenerating, and cultured corneal epithelial and endothelial cells

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The presence of cell-surface receptors accessible to antibodies on regenerating and cultured, but not on resting or metabolically inhibited, corneal epithelial and endothelial cells was established by immunofluorescence or immunocytotoxicity. The metabolically dependent antigens (MDA) can be demonstrated in the corneal cells (membranes) even when absent from the surface of viable cells. This is evidenced by the ability of normal cells to induce an antibody response and by absorption experiments. Thus, corneal regeneration as well as corneal cell culture apparently involves an unmasking of the MDA. In contrast to the restricted specificity of the MDA, the ubiquitous Forssman or blood-group antigens on chicken corneal cells reacted with antibodies independently of their metabolic state. The immunocytotoxic reactions involving Forssman cell-surface antigens were enhanced by complement, whereas, similar reactions involving the MDA were complement independent.

Key words: antibody accessibility, corneal epithelium, corneal endothelium, corneal cell culture, corneal regeneration, cell surface antigens, metabolically inhibited corneal cells, metabolically activated corneal cells, immunofluorescence, immunocytotoxicity.

The aim of this report is to demonstrate that corneal epithelial and endothelial cells contain two classes of cell-surface receptors. Receptors belonging to the first class are present on the cell surface at all times, whereas those belonging to the second class are present on the surface only of actively metabolizing and/or proliferating, but not on the surface of resting, cells. The latter are referred to as metabolically dependent receptors. They occur in vivo, e.g., on cells regenerating after injury or in vitro on cultured cells.1, 2

The presence or absence of cell-surface receptors on normal, regenerating, or cultured corneal cells was established in this investigation by immunocytotoxicity and immunofluorescence tests.

Materials and methods

Immune sera. Antirabbit corneal sera were produced in ducks by intramuscular injections and antichicken corneal sera were produced in rabbits...
Fig. 1. Cytotoxic effects of duck antirabbit corneal antisera (DARCS) on rabbit corneal tissue cultures. Cytotoxic antisera were added to washed tissue cultures and results recorded after three hours of incubation at 37° C. Corneal epithelium cultured three days (A) before and (B) after the addition of DARCS. Corneal endothelium cultured five days (C) before and (D) after the addition of DARCS. Magnification, ×200.

by subcutaneous injections as described earlier.1 All sera used were decomplemented by heat inactivation. Anti-Forssman antibodies were removed from rabbit antichicken corneal serum by absorption with packed sheep red blood cells. Rabbit antiseep hemolysin (Arnel, New York) without preservatives or glycerin and human immune anti-A sera, aseptically prepared,9 were used as anti-Forssman and anti-blood group sera, respectively.

Corneal cultured cells. Corneal epithelial cultures were grown from corneas taken from two- to four-day-old rabbits and 14- to 16-day-old chicken embryos. The corneas, cut into pieces, were explanted in wells of a Microtest Plate (Falcon Plastics Co., Oxnard, Calif.) previously coated with human cord serum. To each well growth medium was added (Medium 199 supplemented with 10 per cent inactivated newborn calf serum, penicillin 100 units per milliliter, and streptomycin 100 μg per milliliter). Corneal epithelial cell cultures were used after three days of growth.

Endothelial cell cultures were grown from corneas of three-week-old albino rabbits. The endothelium was peeled off under a dissecting microscope and placed in 5 ml. of 0.05 per cent (w/v) solution of pronase (B grade, Calbiochem, Los Angeles, Calif.) in Ca++- and Mg++-free Hanks' BSS (BBL, Cockeysville, Md.). After 90 minutes of incubation at 37° C, the dispersed endothelial cells were washed in Hanks' BSS, re-suspended in the growth medium, and planted in wells at the concentration of 5 × 10⁶ cells per milliliter. The cultures were used on the sixth or seventh day of growth.

Corneal regenerating cells. Injury of rabbit corneal endothelium in vivo was done by scraping with a bent needle, and of corneal epithelium by scraping with a curette as described previously.1
Fig. 2. Irreversibility of binding of cytotoxic DARCS to rabbit corneal endothelium in growing culture: (A) normal endothelial monolayer grown from a primary explant, (B) the same monolayer immediately after incubation with DARCS for one hour at 37° C, (C) a ++++ cytotoxic reaction obtained after washing and subsequent incubation of monolayer (b) for three hours at 37° C. Magnification, ×200.

Both corneal tissues were also induced to regenerate by freezing of the corneal surface in vivo for one minute with a CO₂ cryogenic probe (3 mm. in diameter). The ensuing regeneration was followed by the diminution of corneal opacity. Animals were killed when the injured cornea was only very slightly hazy usually on the fifth or sixth day after scraping and on the fourth day after freezing.

**Immunocytotoxicity assay.** Cultures, washed three times with Hanks’ BSS, were treated with two drops of the anticorneal antiserum and incubated at 37° C for three hours. The cytotoxicity was graded from + to ++++ according to the degree of disorganization of the cell sheath and rounding of cells. Normal sera were used as controls.

**Immunofluorescent assay.** Globulin fractions of the immune and normal control sera were conjugated with fluorescein isothiocyanate (FITC) as described earlier, and the conjugates obtained were purified by chromatography on a Sephadex G-25 column (Pharmacia, Piscataway, N. J.) and concentrated by ultrafiltration (Filter PM 10, Amicon Corporation, Lexington, Mass.). The nonspecific staining was removed by absorption with lyophilized mouse liver.

**Fluorescein staining.** For the direct assay, cultures were grown in chamber slides (Lab-Tek Products, Westmont, Ill.). Each culture was washed with Hanks’ BSS and stained with the tagged immunoglobulin (3 mg. per milliliter). After 80 to 90 minutes at 37° C, the slides were washed three to four times with Hanks’ BSS. The slides were examined in a Zeiss Photomicroscope II. Cell cultures exposed to fluorescein-tagged normal globulin served as controls.

In the indirect assay used in the study of blood group cell-surface receptors, chicken corneal epithelial cultures were metabolically inhibited at 4° C. overnight, washed with cold Hanks’ BSS, and exposed to cold human anti-A immune antiserum for four hours at 4° C. The cultures were washed and treated with FITC goat antihuman globulin for 20 to 30 minutes at room temperature. Cell cultures exposed to human AB serum and FITC goat antihuman globulin were used as controls.
To stain normal and regenerating endothelium, corneas were excised, placed in Petri dishes endothelial side up, and treated with labeled antibodies for two hours at room temperature. After washing, endothelial layers were peeled off, mounted on glass slides, and examined.

In case of epithelium, excised corneas were placed in modified Rose chambers and treated with pronase to disperse the cells. The enzyme concentration and incubation time were the same as for tissue culture experiments. The dispersed and washed epithelial cells were then stained with FITC anticomical immunoglobulin.

Results

In vitro cultures of rabbit corneal epithelial and endothelial cells are illustrated in Fig. 1, A and C, respectively. A comparison of Fig. 1, A with Fig. 1, B and C with Fig. 1, D shows that the exposure of these cultures to duck antirabbit corneal serum for three hours results in both instances in a complete disorganization of the cell sheet and a rounding up of cells. This reaction was graded +++ according to the cytotoxic scale used in this report. The above pathological effect of surface-bound antibodies begins first by a retraction of the cell layer. Such morphological expression of the cellular dysfunction starts usually after more than one hour of exposure to the antibodies.

Fig. 2, A and B compare a normal endothelial monolayer before (Fig. 2, A) and after (Fig. 2, B) one hour of incubation with DARCS. There were no significant differences. The binding of antirabbit corneal endothelium antibodies, however, was complete at this time as evi-
Fig. 4. Effect of colchicine (10 μg per milliliter of growth medium) on the sensitivity of corneal endothelial tissue culture to cytotoxic DARCS: (A) endothelial monolayer after a three-hour incubation with colchicine followed by a three-hour incubation with DARCS, (B) the same monolayer near the edge, control endothelium after six hours of incubation with colchicine alone near (C) the explant and (D) periphery. Magnification, x200.

denced in Fig. 2, C by an undiminished ++++ cytotoxic reaction that results when the culture first exposed for one hour to DARCS (see Fig. 2, B) is washed and incubated in fresh medium for an additional two to three hours.

A similar type of experiment was used to demonstrate that irreversible binding of antitissue antibodies occurs only in metabolically active cells. In metabolically inhibited corneal tissue cultures no binding of antitissue antibodies occurs and, consequently, no cytotoxic effect can be observed. Fig. 3 shows the result of such an experiment with rabbit corneal endothelial culture inhibited by cold and Fig. 4 illustrates an experiment using colchicine as an inhibitor. As can be seen in Fig. 3, A, exposure to cold for the experimental period of 24 hours did not visibly change the morphology of the endothelial cell sheet. It also did not change the ability of the cells to react with the anti-endothelial antibodies at 37° C. This is evidenced by the ++++ cytotoxic response to DARCS of an endothelial culture previously exposed to cold (Fig. 3, B). However, when a cold tissue culture is exposed to cold antitissue antibodies at 4° C. for five hours, no visible morphological changes occur (Fig. 3, C). Binding of antibodies also does not occur, as evidenced in Fig. 3, D by the lack of a cytotoxic effect, when the cultures described in Fig. 3, C were washed with cold growth medium and then incubated at 37° C. for three hours.
Fig. 5. Effect of anti-Forsman antibodies on cultures of metabolically inhibited chicken corneal epithelium: (A) normal chicken corneal epithelium, (B) exposed to 4°C for 24 hours, (C) incubated three hours at 37°C with anti-Forsman antibodies without complement, (D) incubated three hours at 37°C with anti-Forsman antibodies and guinea pig complement, (E) cultures precooled to 4°C and exposed to anti-Forsman antibodies for five hours at 4°C, (F) same culture washed, fresh medium, and guinea pig complement added and incubated three hours at 37°C.
Metabolic inhibition by colchicine (10 μg per milliliter) of rabbit corneal endothelium as well as epithelium proved to have an identical effect as cold on the binding of cytotoxic antitissue antibodies to cells. Rabbit endothelial cultures inhibited by colchicine and exposed to strongly cytotoxic antiserum (Fig. 4, A and B) were comparable in morphology to controls with colchicine alone (see Fig. 4, C and D). This indicates a lack of antibody binding in colchicine-inhibited cell cultures.

The presence of metabolically dependent cell-surface receptors present in growing, but not in cold-inhibited, chicken corneal epithelial cell cultures was demonstrated in analogous experiments by the use of rabbit antichicken corneal antiserum from which antibodies to Forssman antigens were removed by absorption with sheep erythrocytes.

The experiment illustrated in Fig. 5 was designed to show that in contrast to the more tissue-specific corneal cell-surface receptors, the Forssman type of chicken corneal cell-surface receptors are accessible to antibodies independently of the metabolic state of the corneal cells. Fig. 5, A shows a three-day-old chicken corneal epithelial tissue culture. Chicken corneal cultures, just like rabbit corneal cultures, are not affected by exposure to cold even for 24 hours (Fig. 5, B). Comparison of Fig. 5, C and D shows that the cytotoxic response to anti-Forssman antibodies is enhanced by complement but occurs weakly also in its absence. The Forssman cell-surface antigens do react with antibodies even in chicken corneal epithelial cultures metabolically inhibited by cold. This metabolically independent, unmasked state of the Forssman cell surface receptors is indicated by a ± cytotoxic effect caused by exposure of cold tissue cultures to cold anti-Forssman antibodies for five hours in the refrigerator (Fig. 5, E). Additional evidence is the strong cytotoxic effect that occurs when the tissue culture is washed and incubated in fresh medium in the presence of guinea pig complement for three hours at 37°C (Fig. 5, F). Chicken corneal tissue was observed to be relatively less sensitive to anti-Forssman antibodies than other chicken tissue cultures, e.g., heart. This may be an indication that there is a low density of Forssman antigens on corneal epithelium. A similar hypothesis concerning transplantation antigens, which also are tissue nonspecific, was made as a possible explanation of the low immunological reactivity of the cornea in transplantation.

This also may explain the relatively weak fluorescent staining of chicken corneal epithelium exposed to human immune anti-A in Fig. 6. The fact that staining was obtained in cold indicates that the accessibility of blood group cell-surface receptors is metabolically independent.

The concept of masked and unmasked membrane antigens was also tested in vivo using corneal cells induced to regenerate. Fig. 7, A shows the very regular beehive-like cellular arrangement of the endothelial surface of a normal rabbit cornea. When such a normal endothelial monolayer was excised and was treated with fluorescein-tagged DARCS globulins for 60 minutes, no binding of fluorescent antibodies could be detected. Regenerating corneal endothelium, however, always showed definite and uniform surface staining with the same antiserum. Fig. 7, B shows that the staining...
was essentially the same uniform surface staining as that seen with actively growing endothelial tissue cultures. The staining appears hazy and weak here because of technical difficulties involving the uniform spreading of excised endothelium on the slide.

Similar fluorescein staining experiments were done with rabbit corneal epithelial cells regenerating in vivo and then dispersed by pronase. Fig. 8 shows that most of the dispersed cells from regenerating corneal epithelium exhibited uniform surface staining (Fig. 8, A and C). In some cases, however, an extremely clear and characteristic patchy staining was observed (Fig. 8, B). Such staining as this was also observed in areas of tissue cultured cells less metabolically active than at the periphery of the monolayer, e.g., closer to the explant. The patchy staining in Fig. 8, B may be indicative of a transitional stage from an unmasked to a masked state of surface antigens. Similarly treated dispersed cells from normal epithelial tissues did not show any staining whatsoever, and neither did con-
controls stained with fluorescein-conjugated normal duck globulin. These experiments also showed that the metabolically dependent antigens in normal corneal epithelium or endothelium in vivo could not be unmasked by treatment with pronase. In all experiments involving immunocytotoxicity or immunofluorescence, suitable controls with normal duck or rabbit serum were employed.

**Discussion**

The existence of antigen determinants which can be detected on excised and dispersed regenerating corneal cells, but not on normal cells treated in the same way, indicates that an activation of cells is a prerequisite for an expression of certain antigenic determinants on cell surfaces. The differences in response to antitissue antisera between growing and metabolically inhibited tissue culture cells further support this view. Normal cells do contain the same antigenic determinants that are expressed on surfaces of activated cells. This is proved by the fact that normal tissue (homogenized) elicits the formation of antibodies capable of interacting with the MDA. Absorption experiments showed that the MDA are localized in cell membranes even when absent from the cell surface. Consequently, the experiments reported here indicate that the immunofluorescent reaction of regenerating or the immunocytotoxic reaction of cultured corneal cells visualize the unmasking of certain cell-surface antigenic determinants rather than their neosynthesis. The expression of the antigen sites on cell surfaces in metabolically stimulated cells may be a result of rearrangements of the cell-surface components as discussed elsewhere. Regeneration may thus be similar to many other phenomena which lead to an alteration associated with the unmasking of receptor sites on the cell surface, e.g., viral infection or neoplasia.

Independent investigations from this laboratory have shown that the MDA of rabbit corneal epithelium and endothelium have different specificities. In the present study, the MDA in the rabbit cornea were demonstrated directly. Forssman type of cell-surface antigens are present in a variety of cells in some animals, e.g., the chicken, but are absent in the tissues of others, e.g., the rabbit. The rabbit cornea does contain, however, other universally distributed cell-surface antigens as transplantation or blood group and perhaps other antigens. The immune response to the cornea as well as to other tissues is dominated by anti-MDA antibodies. Even in a strong rabbit antichicken corneal antiserum the content of anti-Forssman antibodies was low, and the anti-MDA could be demonstrated directly even without prior absorption of the antisera with sheep red blood cells.

In contrast to the restricted specificity of the MDA of the corneal cell surface, the universally distributed Forssman or blood group antigens, when present, were found to be metabolically independent, that is, to be accessible to antibodies in both active and inhibited corneal cells. Furthermore, the immunocytotoxic reaction of corneal cells involving the Forssman antigens was enhanced by complement, whereas a similar immunocytotoxic reaction involving the MDA of the chicken as well as rabbit cornea was not. In this respect, the MDA resemble the Rh cell-surface antigens which also do not bind complement when associated with antibody. The structural relevance of the analogy is under investigation.

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**REFERENCES**


