

Corneal epithelial-specific cell surface antigen recognized by a monoclonal antibody

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SUMMARY

Monoclonal antibodies, specific against cell surface differentiation antigens of human corneal epithelial cells, were developed using epithelial cells resected from human corneas as the immunogens. One of these antibodies reacted specifically with corneal epithelial cells and not with epithelial cells of other tissues when tested by an indirect immunoperoxidase technique. Nonidet P-40 extracts of different subcellular fractions of human corneal epithelial cells were tested for their reactivity against this antibody using an enzyme-linked immunosorbent assay. The results indicated that the antigen recognized by this antibody is associated with the plasma membrane. This was further verified by immuno-electron-microscopic analysis using ferritin-conjugated anti-mouse IgG antibody. This antigen was not detectable in the corneal epithelial cells in primary cultures nor in the epithelial cells from early stages of developing cornea (12 to 18 weeks *in utero*) but was present in the epithelial cells in the corneas of an 8-month-old infant. Therefore, this surface-associated antigen identified in the present study is a developmentally regulated marker of human corneal epithelium.

INTRODUCTION

The human cornea is a multilayered structure consisting of three cellular and two acellular layers. The anterior layer is stratified and is composed of a heterogeneous population of epithelial cells. In the human embryo, the corneal epithelium as well as the epithelium of the conjunctiva, lens and skin are derived from surface ectoderm (Ozanics & Jakobiec, 1982). Initially during foetal development, the corneal epithelium is arranged in two simple cell layers which then undergo stratification until the adult structure of the epithelium is established consisting of five to seven cell layers. From studies of this process in the chick, cell-cell and cell-extracellular matrix interactions have been found to be important for proper stratification (Newsome, 1978; Hay, 1980). These interactions presumably involve cell surface components of the epithelial cells (Hay & Meier, 1976). Studies with rabbit, bovine and primate corneas suggest that this may also be the case in other species, including human (review by Hay, 1980).

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Most investigations of corneal epithelial cell surface have been of a morphological or immunological nature. Manski (1975) has utilized polyclonal and xenogeneic antisera to compare cultured cells with epithelial and endothelial cells in duck cornea. We have utilized the immunological method of Kohler & Milstein (1975) to develop monoclonal antibodies which are specific for antigens associated with human corneal epithelial cell surface. In this communication, we describe the characterization of one specific antibody against corneal epithelial differentiation antigen and also the distribution and localization of this antigen in foetal and adult corneas.

MATERIALS AND METHODS

Hybridoma production

Balb/c mice (Charles River) were immunized with corneal epithelial cells resected from normal human eyes obtained from the Medical Eye Bank of Western Pennsylvania. Approximately 10^5 cells in 0.5 ml of PBS (0.15 M-NaCl, 2.68 mM-KCl, 0.02 M- Na_2HPO_4 , 1.47 mM- KH_2PO_4) was mixed with an equal volume of Freund's complete adjuvant for the first injection and 0.5 ml of this mixture was injected subcutaneously into each mouse. At three-week intervals, second and third injections consisting of 10^5 cells in PBS were given intraperitoneally with and without incomplete adjuvant, respectively. Three days after the final injection, the spleen cells from the immunized mice were fused with mouse myeloma cells, SP2/0 Ag14 (Shulman, Wilde & Kohler, 1978), using polyethylene glycol 1550 (Accurate Chemical & Scientific Corporation, Westbury, NY) at a concentration of 50/50 w/v in Dulbecco's Modified Eagle's Medium without serum (DME-S) (Galfre *et al.* 1977). The fused cells were resuspended in hybridoma media (HY) (Kennett, Denis, Tung & Klinman, 1978) with 20% foetal bovine serum and dispensed into 4×96-well microtitre plates. For the next three days, and every third day thereafter, half of the medium in the wells was replaced with HY medium containing 1.0 μM -hypoxanthine, 0.8 μM -aminopterin and 15.0 μM -thymidine (HAT, Littlefield, 1964). Hybridoma growth was detected within 2–3 weeks after the hybridization.

To facilitate the testing of a large number of hybridomas, a two-step screening procedure was utilized. Culture supernatants were first screened for the presence of antibody by an enzyme-linked immunosorbent assay (ELISA) and IgG-producing hybridomas were then tested for tissue specificity by an indirect immunostaining procedure (see below).

Those hybrids showing appropriate specificity were cloned by limiting dilution so that the average number of clones per well was 0.4. One or two clones were used for further characterization of the antibody. Monoclonal antibodies (MAbs) in culture supernatants of the hybridomas were concentrated by fractionation with ammonium sulphate at 50% saturation. Hybridomas were also grown as ascites tumours in Balb/c mice and the ascitic fluid used as a source of high titres of monoclonal antibodies (McKearn, 1980).

Enzyme-linked immunosorbent assay (ELISA)

An ELISA was performed using rabbit antimouse IgG as the solid substrate coated on 96-well microtitre plates using a modification of the technique of Engvall & Perlmann (1972). These plates were incubated with culture supernatants from hybridomas for 1 h, followed by incubation with horseradish-peroxidase-conjugated rabbit antimouse IgG. The chromogen used was 5-aminosalicylic acid (Voller *et al.* 1978) which turns brown when oxidized. The absorbance was measured in a Titertek Multiscan at 450 nm. This procedure detected both IgG and IgM classes of antibodies because the peroxidase-conjugated anti-IgG used cross reacted with IgM.

Immunostaining

Serial dilutions of ascites fluid containing MAbs, made in PBS with 1% BSA, were tested to select appropriate dilutions for immunostaining. Fresh tissues were frozen in Tissue-Tek II OCT

compound and stored at -70° . $6\ \mu\text{m}$ sections were cut on a cryostat and transferred to 1% gelatin-coated microscope slides for light microscopy. Immunostaining was carried out by an indirect technique using as first antibody the ammonium-sulphate-concentrated medium or ascites fluid from mice bearing tumours of the hybridoma cells, and as a second antibody either a peroxidase-conjugated or a fluorescein-conjugated anti-mouse IgG (SundarRaj, Martin & Hrinia, 1982). To detect binding of the peroxidase-conjugated antibody, a reagent containing benzidine dihydrochloride and safranin O was used (Kaplow, 1965). The sections were dehydrated and mounted for light microscopy. A blue colour product was formed at the site of second antibody binding.

Primary cell culture

Primary cultures of corneal epithelial cells were set up according to the method of SundarRaj, Freeman & Brown (1980). Briefly, corneal explants containing epithelium and some stroma were resected aseptically from donor human (ages 47, 55 and 67) eyes. These explants were placed in 35 mm tissue culture dishes and coverslips placed over the explant. Epithelial cells were allowed to grow out of the explant for 7–10 days and then used for immunostaining.

Determination of immunoglobulin class

Immunoglobulin class and subclass were determined by the double immunodiffusion test (Ouchterlony, 1958) using ammonium-sulphate-concentrated monoclonal antibodies from culture supernatants. These were tested against anti-mouse κ and λ light chains and subclass specific anti-mouse IgG (Miles Laboratories, Inc., Naperville, IL) and anti-mouse IgM (μ chain specific) (Cooper Biomedical, Inc., Malvern, PA).

Cellular fractionation and differential extraction

For the fractionation and differential extractions of corneal epithelial cells resected from donor human eyes, the following procedures were used. The first procedure involved isolation of the plasma-membrane-rich fractions using ZnCl_2 as described by Shin & Carraway (1973). The fractions obtained from the 35–50% sucrose gradient (i.e. bands I through V) were extracted in 0.5% Nonidet P-40 in 25 mM-Tris-HCl buffer, pH 7.4. These extracts were used for coating microtitre wells for an ELISA.

The second procedure involved extracting the cells with 0.025 M-Tris-HCl buffer, pH 7.4 containing 2% Nonidet P-40 or 8 M-urea, 2 mM-EDTA, and 2 mM-phenylmethylsulphonyl fluoride. These extracts were dialysed against 0.025 M-Tris-HCl buffer, pH 7.4 and the dialysates were used to coat microtitre wells for an ELISA.

Immunoelectronmicroscopy

Corneal epithelial cells resected from donor human eyes were washed with PBS, and then incubated with inactivated goat serum for $\frac{1}{2}$ h at room temperature. Following centrifugation, the cells were incubated for 1 h with first antibody, ascites fluid from mice bearing tumours of either hybridoma 1HP3G or SP2/0 Ag14 (control). After washing with PBS, the cells were incubated with a 1:50 dilution of ferritin-conjugated goat anti-mouse IgG (Cooper Biomedical, Inc., Malvern, PA). The cells were washed with PBS, treated with 1% OsO_4 for $\frac{1}{2}$ h, dehydrated in graded ethanols and embedded in Epon-Araldite for electron microscopy.

RESULTS

Production and selection of hybridomas

Human corneal epithelial cells, resected from human donor eyes (ages 65 and 69 years), were used as the source of antigen for immunizing Balb/c mice. From two

separate fusion experiments a total of 300 hybridomas were derived of which 50 secreted IgG or IgM, as determined by the solid phase ELISA described in Materials and Methods. Culture supernatants of these IgG producing hybridomas were then tested for their reactivity with human cornea by indirect immunostaining techniques. A wide variation was exhibited in the patterns of reactivity of these MAbs with human corneal sections. While some MAbs reacted with all five corneal layers, including the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium, others reacted with the components of one or more corneal layers in different combinations. One of these antibodies (designated 1HP3G) reacted only with the human corneal epithelial layer (Fig. 1A). The immunostaining pattern indicated that antigen for this MAb was localized at cell surface regions. Cell surface association of the antigen was more distinct when the immunofluorescence technique was employed (Fig. 1C). Either preimmune mouse serum or a MAb, concentrated from another hybridoma which does not react with corneal antigens, were used as negative controls (Fig. 1B,D). Hybridoma 1HP3G was then cloned and the clones grown for large-scale antibody production.

Determination of antibody class and subclass

Using the double immunodiffusion technique, MAbs secreted by three selected clones of 1HP3G were found to be IgG₁ class with kappa light chains.

Determination of species and tissue specificity

Indirect immunostaining was performed using either a 1:200 dilution of ascites fluid containing antibody 1HP3G or a 1:25 dilution of the antibody concentrated from the culture supernatants. Cryostat sections of corneal tissue from different sources (human, rabbit and mouse) were analysed. This antibody, 1HP3G, reacted specifically with human corneal epithelial cells (Table 1). Sections from other types of ocular tissue including lens, sclera and retina from these three species did not react with 1HP3G. Because epidermal cells are derived embryonically from surface ectoderm as is corneal epithelium, skin from the upper arm, chest and thigh was also tested for their reactivity with 1HP3G. Oesophageal tissue was used as an alternate source of the mucous type of stratified squamous epithelium. None of these epithelia reacted with antibody 1HP3G.

From these data, it appears that the antibody produced by hybridoma 1HP3G is specific for an antigen present only on human corneal epithelial cells.

Expression of 1HP3G antibody-specific antigen on cultured cells

Cells in culture often retain their differentiated functions and hence are useful for *in vitro* studies. Primary cultures of human corneal epithelial cells grown in tissue culture dishes or on coverslips were tested for their reactivity with this antibody. Actively dividing epithelial cells away from the original explant did not

react with 1HP3G (Fig. 2A). However, the nondividing cells in the epithelial sheets of the explant that remained attached to the culture dish reacted very intensely with this antibody (Fig. 2B). Similarly, human corneal epithelial cells grown in monolayer did not react with this antibody.

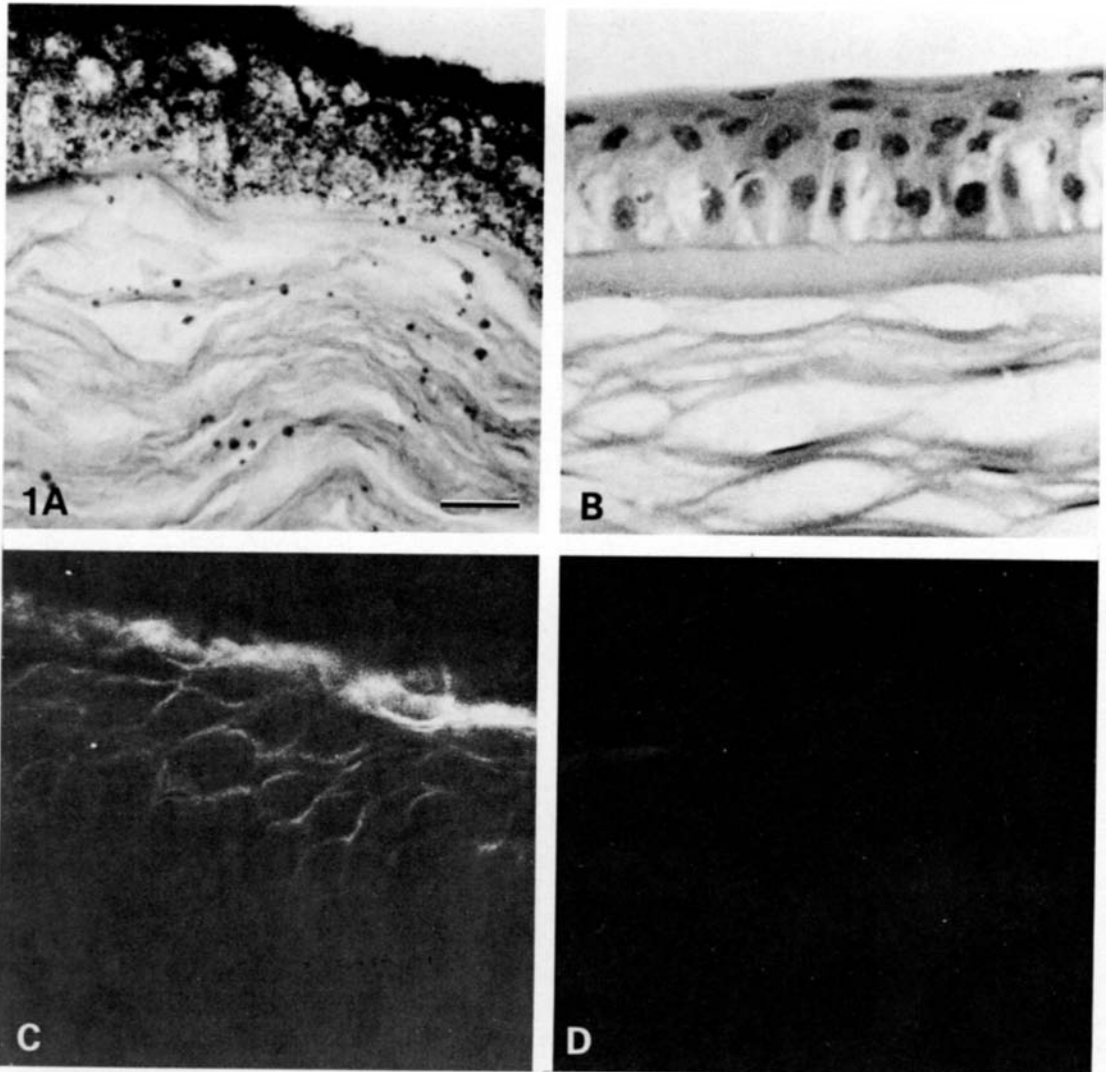


Fig. 1. Indirect immunostaining of human corneal sections. A and C were immunoreacted with monoclonal antibody 1HP3G (concentrated from the culture supernatants); B and D were immunoreacted with concentrated culture supernatants of another IgG producing hybridoma that does not react with the cornea (Control). A peroxidase-conjugated (A,B) or a fluorescein-conjugated (C,D) rabbit anti-mouse IgG was used as the second antibody. A and B were reacted with a reagent containing benzidine dihydrochloride to detect binding of peroxidase-conjugated antibody. A dark reaction is seen at the site of antibody binding in the epithelial region (A). Bar, 20 μm .

Table 1. *Species and tissue specificity**

Antibody	Human			Rabbit			Mouse	
	Cornea	Conjunctiva	Skin	Cornea	Conjunctiva	Skin	Whole Eye	Skin
1HP3G-3D	+	—	—	—	—	—	—	—
7G	+	—	—	—	—	—	—	—
11G	+	—	—	—	—	—	—	—
5/4D†	+	—	—	+	+	—	—	—

* As determined by immunohistochemical staining.
† This is a MAb made against rabbit corneal epithelium, for comparison.
‡ Epithelium.

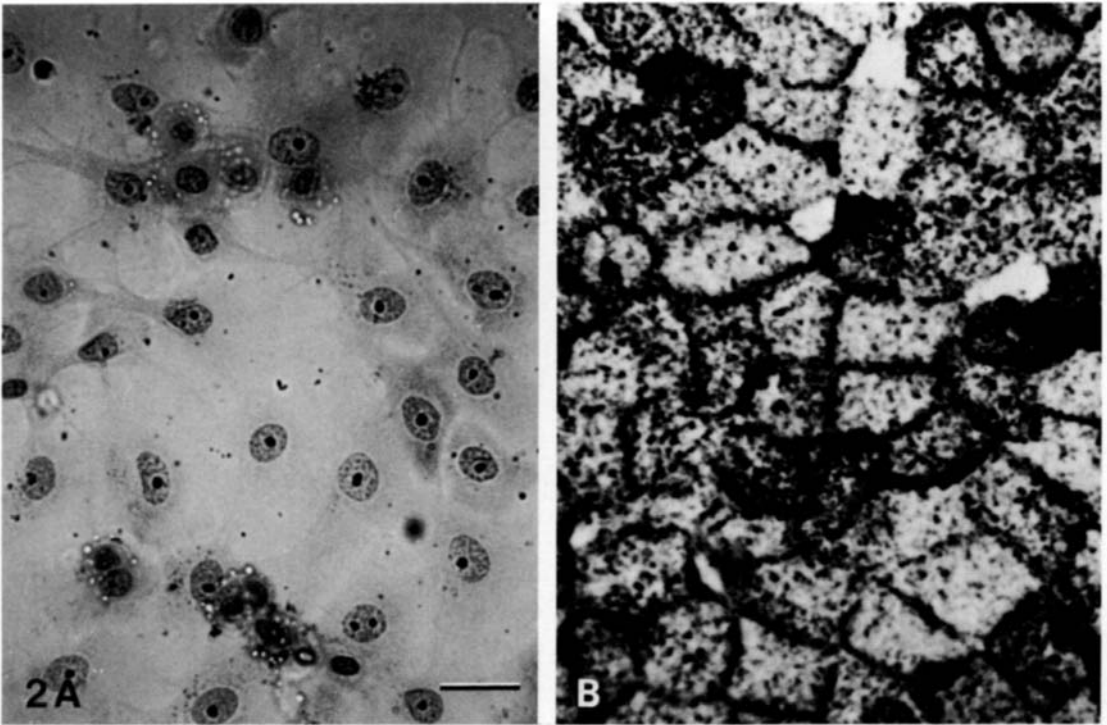


Fig. 2. Indirect immunostaining of dividing corneal epithelial cells in primary cultures (A), and of nondividing epithelial cells in sheets (B) in the same culture dish. The cells were reacted with the ascites fluid containing 1HP3G monoclonal antibody. The second reagent was a peroxidase-conjugated rabbit anti-mouse IgG. A reagent containing benzidine dihydrochloride was used to detect binding of peroxidase-conjugated antibody. A dark reaction is seen at the site of antibody binding (B). Bar, 30 μ m.

Localization of antigen to plasma membrane

The pattern of immunostaining of the epithelium in cryostat sections of the cornea (Fig. 1A,C) strongly suggested that the antigen recognized by 1HP3G was

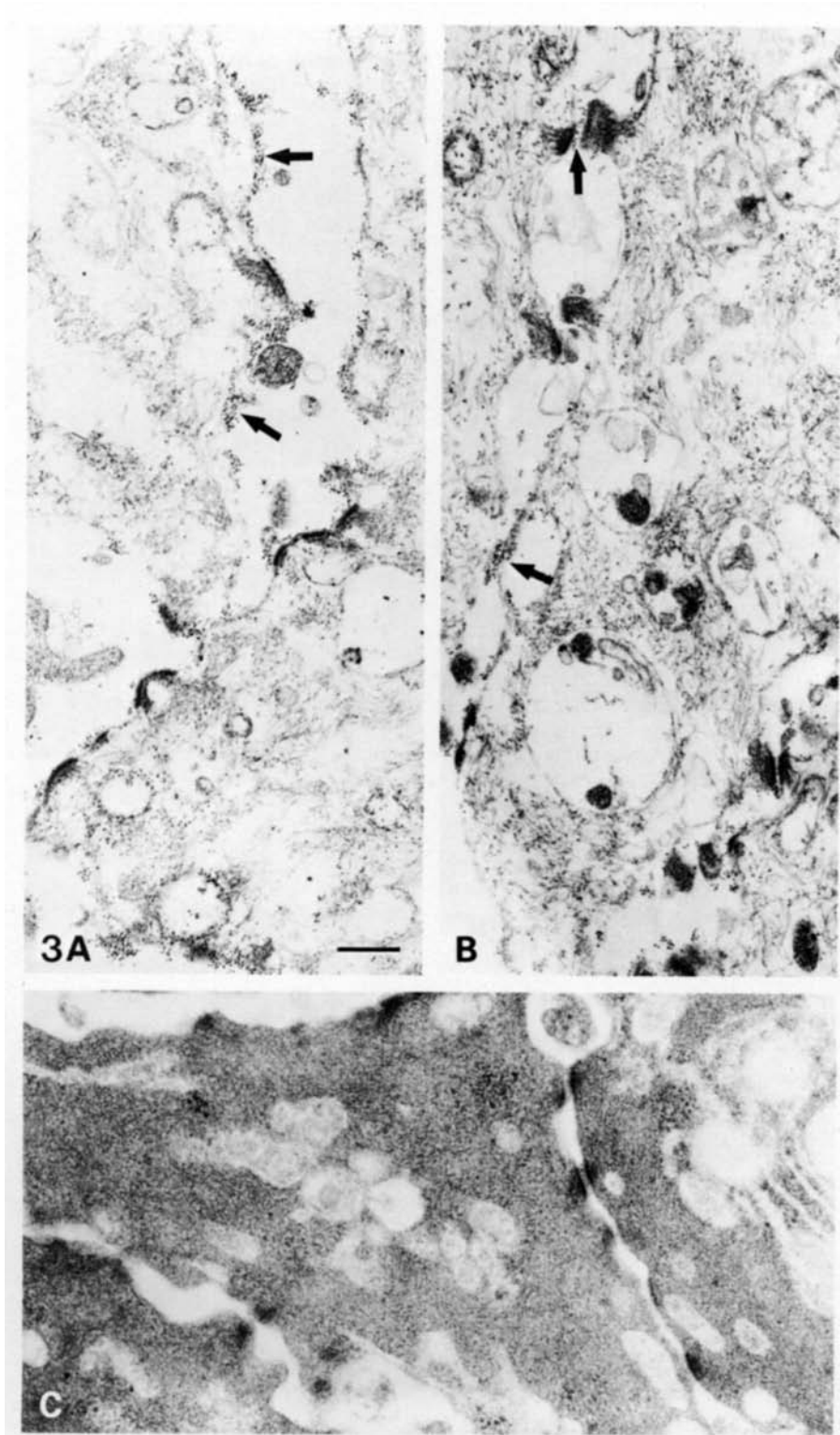


Fig. 3. Indirect immuno-electron microscopy of epithelial cells resected from human cornea. A and B reacted with ascites fluid containing 1HP3G monoclonal antibody; C reacted with ascites fluid from a mouse bearing SP2/O Ag 14 myeloma (control). Ferritin-conjugated anti-mouse IgG was used as the second antibody. Cell surface is seen to be decorated with ferritin granules (arrows). Bar, 20 nm.

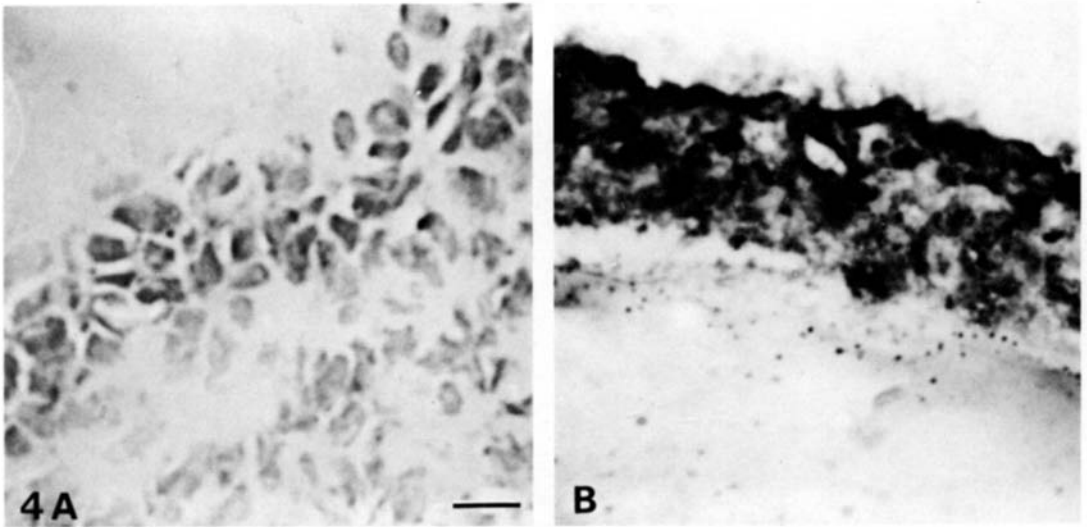


Fig. 4. Indirect immunostaining of human foetal corneal sections. (A) an 18-week-old foetal cornea and (B) an 8-month-old human infant cornea were immunoreacted with monoclonal antibody 1HP3G, concentrated from culture supernatants. A peroxidase-conjugated rabbit anti-mouse IgG was used as the second antibody. A and B were reacted with a reagent containing benzidine dihydrochloride to detect binding of peroxidase-conjugated antibody. A dark-staining reaction is seen at the site of antibody binding in the epithelial region of B. Bar, 20 μ m.

associated with the cell surface. To test whether this antigen was associated with the plasma membrane, subcellular fractions of corneal epithelial cells, separated on a sucrose density gradient, were tested for the presence of the antigen. 1HP3G reacted very strongly with the bands at the interfaces between 35 and 40% and also 40 and 45%, rich in plasma membrane. The antigen was extractable in Nonidet P-40 containing buffer but was not extractable in 8 M-urea. These findings as well the reactivity of the nonfixed cells with the monoclonal antibody led to the conclusion that the antigen of 1HP3G was associated with the plasma membrane.

To further verify this result, immuno-electron-microscopic analysis of human corneal epithelial cells was performed. When 1HP3G was the first antibody, the ferritin-conjugated second antibody was bound to the outer surface of the cells (Fig. 3A,B, arrows). When control ascites fluid was used, there was no binding of ferritin-conjugated second antibody (Fig. 3C). This finding further supported the observation that monoclonal antibody 1HP3G reacted specifically with an antigen associated with the cell surface of human corneal epithelial cells.

Antigen 1HP3G during foetal development

To demonstrate the expression of the antigen for monoclonal antibody 1HP3G, we were able to obtain human foetal corneal specimens to the age of approximately 18 weeks of gestation but no later stages of foetal development. Immunostaining on cryostat sections of these tissues showed no reaction with 1HP3G

(Fig. 4A). A cornea from an 8-month-old infant was the next available stage of postnatal growth. In this cornea, the presence of the antigen for 1HP3G was evident from the positive-staining reaction on the epithelium (Fig. 4B).

DISCUSSION

We have identified and isolated a monoclonal antibody which recognized a human corneal, epithelial-specific, cell-surface-associated antigen. The antigen recognized by this antibody (1HP3G) was present mainly in the membrane-rich subcellular fractions. Its association with the outer cell surface of the plasma membrane, including the desmosomal regions, was evident from immunoelectron microscopic studies. To the best of our knowledge, this is the first report on the identification of a surface-associated corneal epithelial differentiation antigen using a monoclonal antibody.

This membrane-associated antigen was not present on the corneal epithelium during early developmental stages. This observation was not surprising because many changes occur in the corneal epithelium between 12 weeks of gestation and birth (Newsome, 1979). These modifications include increases in stratification and intracellular organelles, the formation of Bowman's membrane and the appearance of attachment complexes at the basal cell membrane. In the explant cultures a central population of nondividing cells only stained with this monoclonal antibody 1HP3G while the rapidly dividing cells at the edge of the explants did not react with this antibody. Therefore, this antibody 1HP3G can be used as a marker for nondividing corneal epithelial cells.

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