Human corneal cells in vitro: morphology and histocompatibility (HL-A) antigens of pure cell populations

David A. Newsome,* Mitsuo Takasugi,**** Kenneth R. Kenyon,* Walter F. Stark,** and Gerhard Opelz***

Pure populations of human corneal epithelial cells, keratocytes, and endothelial cells were established in vitro. Morphologic examination of the cells by phase contrast and electron microscopy showed that, even after multiple serial passages, the cells retained structural specializations typical of their tissues of origin. Using a modified cytotoxic plating inhibition test, HL-A histocompatibility antigens could successfully be detected on all cultured cells. The results of HL-A typing were generally concordant between corneal cells and lymphocytes from the same donor. This study provides direct evidence that HL-A antigens are present on cultured corneal cells, and suggests that such cells may prove useful in the study of immunologic graft rejection encountered in keratoplasty.

Key words: HL-A histocompatibility antigens; tissue culture; electron microscopy; cornea, human; corneal epithelium; corneal keratocytes; corneal endothelium.

Penetrating keratoplasty is highly successful in prognostically favorable cases. Yet, clinically recognizable immunologic graft rejection occurs with significant frequency, particularly in patients with vascularized corneal beds. Experience in man with skin grafts4,5 and, more recently, with renal transplantation6-9 and corneal transplantation10 has indicated that the HL-A antigen system is one of the major transplantation antigen systems in man.6-11 HL-A typing procedures identify cell-surface antigens determined by two closely linked autosomal cistrons.9,12 These two pairs of genes determine the expression of two allelic series of antigens, designated as the "first" and the "second" segregant series. Depending upon homozygosity at these loci, from zero to four different antigens, with a maximum of two from each series, may be detected on the cells.

As a basic step in evaluating the role of HL-A histocompatibility antigens in

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allograft reactions occurring after keratoplasty, we report here the morphologic characteristics of pure cultures of human corneal cells which have been serially propagated in vitro and demonstrate, for the first time, the presence of HL-A antigens in these cells by cytotoxic methods.

Materials and methods

One eye from each of 13 cadavers of persons between 18 and 68 years of age provided the corneal tissue for culture. Using sterile technique, the eyes were enucleated within eight hours after death, and were stored for no more than 32 hours at 4° C. in 0.85 per cent aqueous NaCl before use.

For establishing primary cultures of epithelial and stromal cells, explants measuring about 1 by 2 to 5 mm. of central corneal epithelium, Bowman’s membrane, and superficial stroma (0.3 mm. or less in depth) were prepared with sterile precautions. For explantation of endothelial cells, endothelium with Descemet’s membrane was mechanically stripped free of stroma. Individual explants were rinsed twice in saline G (Fick’s N-15 minus the amino acids)13), placed into separate culture dishes, and squashed gently under autoclaved 13 mm. glass coverslips.

Primary outgrowths of cells were serially propagated after they had attained diameters of 10 to 20 mm. These outgrowths were propagated by washing in Ca++- and Mg++-free phosphate-buffered saline,14 and by incubating them for 20 minutes at 37.5° C. in 1 ml. of Coon’s enzyme solution (6 units of collagenase per milliliter, 0.1 per cent weight per volume of trypsin, and 2.0 per cent of chick serum15) with 4 mM EDTA for epithelial and endothelial cells, and without EDTA for keratocytes. Suspended cells were then pipetted into new dishes containing growth medium. At least one uncloned population of each epithelial cells and of keratocytes was propagated from each cornea.

Both primary and serially propagated cultures were maintained either in 60 mm. diameter plastic tissue-culture dishes or in 30 ml. plastic tissue-culture flasks (Falcon Plastics, Inc.) containing 3 ml. of growth medium. The modified Ham’s F-12 growth medium (pH 7.2) with 5 per cent virus-screened fetal calf serum (GIBCO) and 3 μg of penicillin per milliliter15 was changed every 3 days. Cultures were kept in a moist chamber incubator at 37.5° C. in a 5 per cent CO₂ and 95 per cent air atmosphere.

The “plating efficiency” was defined as the percentage of cells seeded which had attached to the culture dish six to eight hours after seeding. The rate of proliferation of a cell population was determined by seeding known numbers of cells (usually 10³ to 5 × 10⁴) into replicate dishes, then at daily intervals suspending the cells present in pairs of dishes with Coon’s enzyme solution and counting them. The number of cells present was routinely determined by duplicate hemocytometer counts.

Living cells were observed and photographed through a WILD phase-contrast inverted microscope. For electron microscopy, cells from serially propagated populations were fixed and embedded in the culture dish by techniques previously described,16 and were observed with a JEM 100-B electron microscope.

HL-A antigens on the cultured corneal cells were determined by a cytotoxic plating inhibition test.17 This test involved reacting known anti-HL-A sera against cultured target cells in the presence of complement. Cell viability is then determined by the ability of the cells to plate onto plastic surfaces. A positive reaction results in death of the target cells and inhibition of cell plating. For four of the donors, lymphocytes were also HL-A types by the microlymphocyte cytotoxicity test.18 In addition to the 27 different HL-A antigens detectable on human lymphocytes that are officially recognized,19 we also tested for additional antigens recognized in our laboratory. All nomenclature used in this communication is, however, standard.20

Results

A. Explant success. Between 16 and 30 explants of corneal epithelium and stroma were established for each eye. Of the 320 total explants, 267 gave rise to cellular outgrowth (83 per cent success rate). In sharp contrast, 152 endothelial explants (10 to 16 from each eye) produced cellular outgrowth in only eight instances (5 per cent success rate). In all cases, explants were established between 7½ and 40 hours postmortem. This variable did not significantly affect the success rate of epithelial, keratocytic, or endothelial outgrowths. Donor age also appeared to have no effect on the success of cellular outgrowth. No cultures had visible bacterial or fungal contamination.

B. Characteristics of cells obtained from explants. Within 12 to 30 hours in vitro, a compact monolayer of polygonal cells with a typically epithelioid appearance surrounded the explant (Fig. 1, A). Keratocytic cells did not migrate out of the explant before five days in culture (range: 5
Fig. 1. Appearance of primary outgrowths from corneal explants. A, after three days in vitro, corneal epithelium forms a compact monolayer of polygonal cells extending from the explant (X). B, after six days, long, thin keratocytes have emerged from the explant and have overgrown the epithelial sheet. C, by 10 days in culture, a uniform population of keratocyte cells have grown out of this explant. (Phase contrast, X560.)
to 10 days), and could then be seen overlying the epithelial sheet (Fig. 1, B). Keratocytes were distinguished by their spindle shape and growth in layers of closely packed cells with their long axes in parallel (Fig. 1, C). Removal of the explant prior to the appearance of the keratocytes left a sheet of cells that by phase-contrast microscopy appeared free of stromal cells. Once keratocytes invaded the primary outgrowth, however, epithelial cells were overgrown in three to four days and the two cell types could not be separated in subsequent cultures.

In contrast to the rapid outgrowth of epithelium and keratocytes, endothelial explants from only three eyes gave rise to cellular outgrowths that appeared only after 14, 28, and 31 days in vitro. They appeared as flat rhomboidal cells forming a loose monolayer (Fig. 5, A). From these outgrowths, only two cell populations were established.

C. Plating efficiency and rate of proliferation of passed cells. Homogeneous primary outgrowths of either epithelial, keratocytic, or endothelial cell populations were serially propagated as uncloned cultures. The plating efficiency varied among the three cell types. Keratocytes had the highest average plating efficiency, 78 per cent (range: 64 to 90 per cent), followed by endothelium, 62 per cent (56 per cent and 68 per cent), and epithelium, 57 per cent (range: 51 to 74 per cent).

The rate of proliferation of the different cell populations also varied according to cell type (Fig. 2). Keratocytes proliferated most rapidly, followed by epithelial cells and endothelial cells.

D. Morphology of serially propagated cells. Serially passed epithelial cells generally retained an epithelioid morphology (Fig. 3, inset). By electron microscopy (Fig. 3), several ultrastructural characteristics of corneal epithelial cells in vivo were...
Fig. 3. Inset: this phase-contrast photomicrograph shows the retention of epithelioid morphology by corneal epithelial cells after seven serial passages. (x560.) By electron microscopy, corneal epithelial cells (after six passages) are distinguished by microplicae-like foldings (circled) of the free surface membrane, fine tonofibrils (F) in the apical cytoplasm, and numerous well-defined desmosomes (arrows). (x8,500.)

maintained by the propagated cells, including: (1) formation of multiple (usually two to three) layers of squamous cells, (2) folding of free surface plasma membranes into microplicae, (3) appearance of a fine tonofilament network in the apical cytoplasm, and (4) development of numerous well-defined desmosomal attachments between adjacent cells. No basement membrane or fibrillar material could be identified in the extracellular spaces.

Propagated keratocytes appeared typically fibrocytic, and tended to pile up in multiple layers of orthogonally apposed cells; that is, the long axes of the cells in one layer were oriented at nearly right angles to the long axes of cells in adjacent layers (Fig. 4, inset). Electron microscopy of these cells revealed extensive dilated cisternae of rough-surfaced endoplasmic reticulum, but no intercellular attachment specializations (Fig. 4). They had deposited large masses of extracellular fibrils (presumably collagenous) with diameters of approximately 100 to 150 A.

Serially propagated endothelial cells appeared to be flat, polygonal, and arranged in compact monolayers under phase-contrast microscopy (Fig. 5, B). Ultrastructural examination showed monolayers or bilayers of flattened cells with a definite polarity as evidenced by well-developed apical junctional complexes (Fig. 5, C). The extracellular space contained fine fibrils measuring less than 100 A in diameter with no resolvable macroperiod. This fibrillar substance could not be distinguished from basement membrane material.

E. HL-A typing. All successfully cultured cell populations could be HL-A typed by our methods (Table I). From one to four antigens were typed in each cell population, with no more than two antigens detectable in each of the two segregant series of the HL-A system. The typing of the cultured cells generally agreed with the lymphocyte typing in the cases where the lymphocytes also were tested (B. H., S. S., L. W., and A. S.; Table I), except that
Fig. 4. Inset: this phase-contrast photograph of keratocytes following five serial passages demonstrates the orthogonality of alternating layers of spindle-shaped cells. (×240.) By electron microscopy, multiple layers of keratocytes (following eight passages) suggest this orthogonal arrangement of these fibroblastic-appearing cells which contain extensive dilated cisternae of rough endoplasmic reticulum (circled) and have deposited masses of extracellular fibrils (*), but have not formed intercellular attachments. (CD: culture dish; ×8,000.)
Fig. 5. Appearance of corneal endothelial cells in vitro. A, primary endothelial outgrowth after 31 days in culture consists of large polygonal mononuclear cells. (Phase-contrast, ×460.) B, after eight serial passages, these endothelial cells formed a compact layer of mononucleated polygonal cells. (Phase-contrast, ×460.) C, electron microscopy of serially passed endothelium (eight passes) reveals a bilayer of squamous cells with apical junctional complexes (arrows) and fibrillar material (*) between their apposed basal surfaces. (CD: culture dish; × 8,500.)
fewer antigens were usually detected in the corneal cells. When more than one type of cultured cell from the same cornea was tested, most antigens were found to be concordant.

Cell populations in several instances were typed after a brief period in culture and again after three to nine additional serial passages. In such cases (Table I: B. H. and L. W., for example), prolonged time in vitro did not correlate with any consistent shift in the number of detectable antigens. Although antigens in lymphocytes tend to change after several subcultures, we did not observe this in corneal cells. Indeed, the specific HL-A antigens which could be detected on any given cell population appeared to be independent of time in culture.

**Discussion**

HL-A antigens are believed to be the major known transplantation antigens in man, and as such have been clearly implicated in the immunologic rejection of skin and kidney allografts. Preliminary work of Ehlers and Ahrons indicated the presence of HL-A antigens on ground-up corneal tissue by nonspecific absorption studies, and recently Stark and co-workers have demonstrated that, in patients with vascularized corneal beds, immunologic rejection of corneal allografts was accompanied by the production of humoral antibodies against HL-A antigens.

Since the HL-A antigen system is genetically determined, it is not surprising that HL-A antigens should occur on corneal cells. Moreover, the close concordance be-

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**Table I. HL-A histocompatibility antigens detected on lymphocytes and on serially propagated human corneal cells in vitro**

<table>
<thead>
<tr>
<th>Donor</th>
<th>HL-A Series</th>
<th>Lymphocytes</th>
<th>Corneal epithelium</th>
<th>Keratocytes</th>
<th>Corneal endothelium</th>
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<td>No. serial passages</td>
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<td>B. H.</td>
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<td>9-11</td>
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<td>2,12, —</td>
<td>2,12</td>
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<tr>
<td>S. S.</td>
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<td>5,32</td>
<td>5,32</td>
<td>32</td>
<td>2,29</td>
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<td>28,10</td>
<td>28,10</td>
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<td>2,29</td>
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<tr>
<td>A. S.</td>
<td>1</td>
<td>2,29</td>
<td>2,29</td>
<td>11,21</td>
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<td>L. W.</td>
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<td>—,28</td>
<td>2,30</td>
<td>—,8</td>
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*A blank (—) indicates that no currently recognized HL-A antigen was detected in that segregant series.*
between the antigens detected on lymphocytes and cultured corneal cells from the same donor leaves no doubt that HL-A antigens are specifically present on corneal cells. A modified version of the conventional HL-A typing method is, however, necessary to detect these antigens on cultured cells. Conjoint epithelium and fibrocytes cultured and HL-A types by the techniques described in this communication showed detectable HL-A antigens in close concordance with those found on the corneal cells from the same person (Newsome and Takasugi, unpublished observations). What disagreement was observed among cell populations from the same donor may have been due either to cross-reactivity within the HL-A system, or to variations in the sensitivity of different cell lines to complement-dependent cytotoxicity. Improved typing of cultured cells and closer agreement among different cultures might be obtained by predetermining that the serum used for typing is not itself cytotoxically reactive against the cultured cells.

Our techniques of cell culture, by taking advantage of the temporal separation of epithelial and keratocytic outgrowth from corneal explants, allowed the establishment of pure populations of these cell types in vitro. In attempting to attribute specific immunologic properties to particular cell types, it is essential to establish with certainty the identities of explanted tissues and serially propagated cell lines. Our morphologic evidence from phase-contrast and electron microscopy, indicating that cultured corneal cells, even after multiple passages, retained the specialized characteristics of their tissues of origin, seemingly fulfills this requirement and thereby assures the specificities of HL-A antigens associated with each cell type.

In summary, the present work demonstrates the feasibility of maintaining serially propagated populations of corneal cells that in vitro retain an epithelial, keratocytic, or endothelial morphologic identity. Further, HL-A antigens could reliably be detected on these cells by the cytotoxic plating inhibition test, and, in those cases tested, were in generally close agreement with the HL-A lymphocyte antigen type from the same donor. The availability of such cells with their associated antigens from donor corneas should make it possible to learn more about immune sensitization by the donor graft in penetrating keratoplasty, and then to explore further the importance and role of HL-A antigens in the phenomenon of corneal allograft rejection.

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REFERENCES