Retinal Pigment Epithelial Cells In Post Mortem HLA Typing Of Corneal Donors

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Corneas used for transplantation are typically obtained from donors up to 48 hr post mortem. By this time, standard HLA typing usually is impossible because of the lack of viable lymphocytes in spleen and peripheral blood. To increase the number of HLA-typed corneas, we developed a method in which the retinal pigment epithelial (RPE) cells of the donor eye are isolated, cultured in the presence of 1000 IU/ml interferon-gamma (IFN-γ), and, after 4 d, are typed for HLA Class I and Class II antigens in the standard NIH cytotoxicity assay. Sixty five donors were typed simultaneously using peripheral blood lymphocytes and RPE. One hundred and sixteen out of 120 HLA-A antigens, 125/127 HLA-B, 106/108 HLA-C, and 92/100 HLA-DR antigens were identical using the same technique. Donor age, sex, cause of death, time of enucleation post mortem, and time of RPE preparation post mortem, as well as duration of culture period prior to stimulation with IFN-γ did not correlate with the results of HLA typing. These data show that RPE cells can substitute for lymphocytes in post mortem HLA typing. Consequently, every donor with corneas suitable for transplantation can be prepared for matched transplantation. Invest Ophthalmol Vis Sci 33:1940-1945, 1992

The overall success rate for corneal transplantation is over 90%. However, high-risk recipients have an estimated 5 yr failure-free graft survival of approximately 50%, which is worse than the rates for recipients of renal, heart, and liver transplants.1-2 Numerous investigators have suggested that donor-recipient HLA matching may improve graft survival in patients at high risk for corneal allograft failures.3-11 Nevertheless, the transplantation of histocompatible corneas has been hampered mostly by logistical problems, such as the use of short-term culture for corneal preservation, prolonged waiting time for the patient, increased costs, and most severely by the limited number of tissue-typed grafts available.

Almost every cornea with a known HLA type originates from multi-organ donors. In contrast, most corneas available for transplantation in Europe and the United States are procured from other donors as whole globes or corneas with a blood sample for HIV and hepatitis screening up to 48 hr post mortem. By this time, conventional, serological HLA typing often is no longer possible because spleen and blood lack sufficiently viable lymphocytes. On the other hand, the need for matched grafts is steadily increasing as more transplants are performed and the proportion of high-risk recipients increases. We sought to develop an alternative method of HLA typing that could be applied to ocular tissue obtained as long as 48 hr post mortem.

We selected retinal pigment epithelial cells (RPE) as the source for the cytotoxicity assay because of their easy isolation with a high yield of viable cells and their rapid growth in tissue culture. Because the expression of a critical density of major histocompatibility complex (MHC) antigens is a prerequisite for successful serological typing, the culture media was supplemented with 1000 IU/ml interferon-gamma (IFN-γ) and a combination of 500 IU/ml IFN-γ plus 100 ng/ml tumor necrosis factor-alpha (TNF-α). IFN-γ is known to enhance and induce expression of MHC gene products in a dose dependent manner.12,13 TNF-α is reported to have an enhancing effect on constitutive and IFN-γ-induced HLA Class I/II cell surface antigen expression.14,15

Materials and Methods

Preparation of RPE

RPE cell suspensions were prepared as described recently.16 The corneoscleral disk was removed under sterile conditions and transferred to organ culture for later transplantation. After removal of lens and iris, the residual eye cup was sectioned by two longitudinal
incisions toward the optic nerve. Gentle rinsing with Dulbecco’s phosphate buffered saline (PBS), Ca²⁺- and Mg²⁺-free (Gibco, Paisley, UK), allowed prompt separation of the vitreous and neural retina from the layer of RPE and allowed the detachment of the choroid from the sclera. The RPE adhering to Bruch’s membrane on the obtained choroidal sheets were washed in PBS and treated repeatedly (three to four times) with 0.025% trypsin-EDTA solution (Gibco) for 10 min at 37°C. The isolated cells were centrifuged at 300 × g for 15 min, resuspended in RPMI 1640 (Gibco), supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK), seeded in six-well culture plates (Costar, Cambridge, MA), and incubated at 37°C in a humidified atmosphere with 5% CO₂. After 24 hr, nonadherent cells were removed and the medium was refreshed. Adherent cells showed growth characteristics that have been described previously.¹⁷⁻²⁰

Induction of HLA Antigen Expression

The culture period required for the earliest clearcut definition of HLA antigens was determined as follows. RPE cell samples from a donor (four different donors were tested) were cultured in the presence of a single dose of 1000 IU/ml IFN-γ and in the presence of single doses of 500 IU/ml IFN-γ plus 100 ng/ml TNF-α, respectively, for 1, 2, 3, 4, 5, and 6 d. (Recombinant human cytokines were a gift from Dr. G. Adolf, Bender Inc., Vienna, Austria). Sequential HLA-typing was performed for each single day of incubation.

HLA Typing of RPE

For the typing procedure, RPE were harvested after a brief incubation with 0.025% trypsin EDTA solution. The cytotoxicity assay with carboxy-fluoresceindiacetate (C-FDA; Sigma, St. Louis, MO) was performed using RPE instead of platelets.²¹ For staining of viable cells, 1 × 10⁶ cells were placed in 0.5 ml PBS plus 10 µl C-FDA stock solution (10 mg C-FDA dissolved in 1 ml aceton) for 15 min at 37°C, then washed twice in PBS and adjusted to a final concentration of 2500 cells/µl PBS. HLA typing was performed according to the standard technique of the NIH microlymphocytotoxicity assay.²² Briefly, 1 µl cell suspension plus 1 µl antisera were incubated for 30 min at 22°C. Thereafter 5 µl rabbit sera, as a source for complement, were added, and the results were evaluated after incubation periods of 1, 2, 3, 4, 5, 6, 12, and 18 hr under the inverted fluorescence microscope. With this method, viable cells fluoresce green, whereas dead cells are only visible under phase contrast conditions. All antisera stemmed from the last four International Histocompatibility Workshops.²³,²⁴

HLA Typing of Lymphocytes

Lymphocytes were prepared according to standard techniques²³ from heparinized blood samples obtained intra vitam or immediately after the death of the donor. HLA typing was performed with the same HLA antisera used for typing of RPE using the standard NIH microlymphocytotoxicity assay.²² All samples were processed and typed in a masked fashion.

Quantitation of Antigen Expression

For the quantitation of HLA-ABC antigen expression, sequential HLA typing was performed for each single day of incubation. Twenty antisera corresponding to the HLA-ABC type of each RPE and 15 HLA-DR antisera were used. The reactions in the cytotoxicity assay were read after 4 hr of incubation with rabbit serum, and the percentage of killed cells was evaluated. The induction of HLA antigens was deduced from the difference in the percentage of killed RPE by the specific antisera and the percentage of killed RPE in sera containing no HLA antibodies (this latter value was always <15%). The mean increase of the percentage of killed RPE was calculated by the formula:

\[ \Sigma (\text{specific killing} - \text{unspecific killing})/n \]

Specific killing = percentage of dead RPE in specific HLA antisera; unspecific killing = percentage of dead RPE in sera without HLA antibodies; n = number of HLA antisera used. (Fig. 1).

Donor Data

Sixty-five donors (42 male, 23 female), from which a reliable HLA type had been obtained from peripheral blood or spleen lymphocytes, were selected for RPE isolation and typing with the same technique. Causes of death were traumatic head injury (n = 36), intracranial hemorrhage (n = 11), rupture of aortic aneurysm (n = 2), neoplasm (n = 7), pulmonary embolism (n = 2), myocardial infarction (n = 2), biliary cirrhosis (n = 1), rejection of an organ graft (n = 2), and gastric ulcer hemorrhage (n = 2). Donor age ranged from 5–78 yr (mean 39.69, standard deviation 18.67, standard error of the mean 2.32). Eyes were enucleated between 0.1 and 42 hr post mortem (mean 5.38, SD 8.20, SEM 1.02) and RPE cells were isolated as described above between 5 and 92 hr post mortem (mean 29.48, SD 24.03, SEM 2.98). Based on the results from the preliminary experiments (Fig. 1), culture medium was supplemented with 1000 IU/ml
IFN-γ 4 d before HLA-typing. Prior to the addition of IFN-γ, RPE had been in culture for 1–78 d (mean 25.60, SD 17.15, SEM 2.11).

Results

RPE cultures could be established from all 65 donors, independently of donor age, cause of death, enucleation time post mortem, and preparation time of RPE post mortem. The isolation technique yielded on average 2 × 10^6 RPE cells from paired globes. Conventional HLA typing of freshly isolated RPE cells, or cells cultured in the absence of cytokines, usually did not allow a clear-cut definition of HLA-ABC (Class I) antigens, and HLA-DR (Class II) gene products could not be defined at all. Expression of these antigens, however, could be enhanced readily with the addition of cytokines. Determination of culture conditions under which RPE cells express HLA Class I and Class II antigens in sufficient density to permit serologic HLA typing is shown in Figure 1.

Sequential HLA typing was performed with RPE cells cultured in the presence of a single dose of 1000 IU/ml IFN-γ, and in the presence of a combination of 500 IU/ml IFN-γ plus 100ng/ml TNF-α for 1 to 6 days. Serologic HLA typing was sequentially performed for each day of incubation. HLA-ABC (Class I) and HLA-DR (Class II) antigen expression are expressed as mean increase of percentage of killed RPE calculated by the formula: \[ \Sigma = \frac{\text{specific killing} - \text{unspecific killing}}{n} \]. Specific killing = percentage of dead RPE in specific HLA sera; unspecific killing = percentage of dead RPE in sera without HLA antibodies; \( n \) = number of HLA antisera used.

Class I could be identified after 24 hr, but the HLA-DR phenotype could not be determined through 6 d of culture (Fig. 1). Analogous results were obtained with the three other donors tested. Based on these data, RPE were incubated for 4 d with a single dose of 1000 IU IFN-γ/ml in further HLA typing experiments. Sixty-five corneal donors, from which an HLA phenotype had been obtained intra vitam or immediately post mortem with peripheral blood or spleen lymphocytes in the serologic NIH microlymphocytotoxicity assay, were selected for simultaneous typing from RPE. In 12 donors, HLA-DR antigens could not be defined because of poor viability of lymphocytes (six donors) or RPE (six donors). HLA antigens obtained from lymphocytes were taken as the standard. HLA antigens obtained from RPE were considered discrepant when, compared to lymphocytes, antigens were either missing, different, or additionally present. Sixteen (3.5%) of 455 antigens defined on lymphocytes were discrepant on RPE (Table 1). Four out of 120 were HLA-A, 2/127 were HLA-B, and 8/100 were HLA-DR specificities.

In 12 (18.46%) out of 65 donors, discrepant assignment of HLA antigens occurred. Table 2 shows the phenotypes of those donors. Donor age, ranging from 5–78 yr (mean 39.69, SD 18.67, SEM 2.32), did not correlate with the successful detection of MHC gene products (\( r = 0.08 \)). Furthermore, no correlations were found between typing results and sex (42 male, 23 female; \( r = 0.03 \)), cause of death (\( r = 0.01 \)), hours of enucleation post mortem...
Table 1. Overall comparison of HLA antigens obtained from RPE and peripheral blood lymphocytes

<table>
<thead>
<tr>
<th>HLA locus</th>
<th>Identical</th>
<th>Discreant</th>
<th>Antigens defined</th>
<th>Donors tested</th>
<th>&quot;Homozygotes&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>116</td>
<td>4</td>
<td>120</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>HLA-B</td>
<td>125</td>
<td>2</td>
<td>127</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>HLA-C</td>
<td>106</td>
<td>2</td>
<td>108</td>
<td>65</td>
<td>22</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>92</td>
<td>8</td>
<td>100</td>
<td>65</td>
<td>6</td>
</tr>
</tbody>
</table>

HLA antigens obtained from lymphocytes were taken as the standard. HLA antigens obtained from RPE were considered discrepant when, compared to lymphocytes, antigens were either missing, different, or additionally present. Sixty-five donors were typed simultaneously from RPE and lymphocytes for HLA Class I and Class II antigens in the NIH-microlymphocytotoxicity assay. Four days before typing RPE cell samples were pretreated with a single dose of 1000 IU/ml IFN-γ.

(0.15–42 hr, mean 5.38, SD 8.20, SEM 1.02, r = 0.11), time of RPE cell preparation post-mortem (5–92 hr, mean 29.48, SD 24.03, SEM 2.98, r = 0.26), and interval of culture prior to stimulation with IFN-γ (1–78 d, mean 25.71, SD 17.0, SEM 2.11, r = 0.22).

Discussion

Numerous investigators have found that donor-recipient HLA matching improves corneal graft survival in patients at high risk for allograft failure.3–11 The vast majority of cornea donors in Europe and the United States cannot be typed by conventional serologic means from lymphocytes, because by the time of enucleation (up to 48 hr post mortem) lymphocyte viability is poor. Immunosuppressive therapy may further reduce lymphocyte viability in donors treated for neoplasms. The need to identify an alternate, readily available source of cells for typing is obvious.

Some investigators have used DNA-hybridization techniques to define HLA restriction fragment length polymorphism instead of the serologic cytotoxicity assay for post mortem typing.12 The method, however, requires experience in molecular biology techniques and takes about 2 wk to provide a final result. In addition, determination of the phenotype is limited to the definition of HLA Class II antigens.17

In our previous experiments that used RPE as an alternative cell type to lymphocytes in the cytotoxicity assay, the interpretation of results frequently was difficult because the expression of a critical density of MHC antigens is a prerequisite for successful serologic typing.16,18 IFN-γ is known to enhance and induce expression of MHC gene products in a dose-dependent manner in a variety of cells,12 including RPE.13 TNF-α by itself is not able to induce HLA-DR expression, but it has been reported that TNF-α has an enhancing effect on constitutive and IFN-γ-induced HLA Class I/II cell surface antigen expression.14,15 However, in the experiments described above, substantial enhancement was only noticed for Class I antigens, whereas Class II antigen expression, upon combined treatment with the two cytokines, obviously did not reach critical density for serotyping. The optimal time for complete HLA phenotype analysis in our setting was found to be the 4th and 5th day of incubation with a single dose of 1000 IU/ml IFN-γ.

Sixty five donors were typed simultaneously from

Table 2. Comparison of HLA phenotypes obtained from lymphocytes and RPE of 12 donors in which discrepant* assignment of antigens occurred

<table>
<thead>
<tr>
<th>Donor initials</th>
<th>HLA antigens on lymphocytes</th>
<th>HLA antigens on RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WK</td>
<td>A1,3;B7,21(49);Cw7;DR3,w6</td>
<td>A1,3;B7,21(49);Cw7;DR−,w6</td>
</tr>
<tr>
<td>GG</td>
<td>A2,11;B8,35;Cw4,w7;DR3</td>
<td>A2,1;B8,35;Cw4,w7;DR3</td>
</tr>
<tr>
<td>TF</td>
<td>A1,3;B35;Cw4</td>
<td>A11,3;B35;Cw4</td>
</tr>
<tr>
<td>KU</td>
<td>A1,9(24);B35,62;Cw3,w4;DR1,w5</td>
<td>A19(24);B35,62;Cw3,w4;DR1,w4</td>
</tr>
<tr>
<td>WC</td>
<td>A2,9(23);B13,27;Cw1,w6,DRw6,w9</td>
<td>A3,9(23);B13,27;Cw1,w6,DRw6,−</td>
</tr>
<tr>
<td>HM</td>
<td>A3;B12,14;Cw5</td>
<td>A3;B12,14;Cw5</td>
</tr>
<tr>
<td>NK</td>
<td>A2,9(24);B15(62),w4;Cw3,DRw6,−</td>
<td>A2,9(24);B15(62),w4;Cw3,DRw6,5</td>
</tr>
<tr>
<td>HW</td>
<td>A9(24),11;B18,16(38);Cw7,DR5,−</td>
<td>A9(24),11;B18,16(38);Cw7,DR5,3</td>
</tr>
<tr>
<td>WA</td>
<td>A9(24),11;B14,27;Cw1,w2;DR1,−</td>
<td>A9(24),11;B14,27;Cw1,w2;DR1,7</td>
</tr>
<tr>
<td>DM</td>
<td>A2,11;B35(51),15(62);Cw3;DR4,w8</td>
<td>A2,11;B35(51),15(62);Cw3;DR4,5</td>
</tr>
<tr>
<td>HH</td>
<td>A2,9(24);B7,35;Cw3,w7;DR1,2</td>
<td>A2,9(24);B7,40(60);Cw3,w7;DR1,2</td>
</tr>
<tr>
<td>PL</td>
<td>A9(24),19(30);B12(44),13;Cw6;DR4,7</td>
<td>A9(24),19(30);B37,13;Cw6;DR4,7</td>
</tr>
</tbody>
</table>

* Discrepant: antigen either missing, different, or additionally present on RPE.
lymphocytes and IFN-γ-stimulated RPE. When comparing the results, 16 (3.5%) out of 455 antigens defined on lymphocytes were discrepant on RPE (Table 1). The discrepancies were observed mainly with antigens known to cross react with alloantisera, eg, between antigens A1 and A11 (donors GG, TF, and KU in Table 2). Cross reactivity also is held responsible for discrepancies observed between Cw5 and Cw8 (donor HM), as well as between HLA-DR5 and DR4 (donor TF), DR5 and DRw8 (donor DM), DR3 and DRw6 (donor NK), and between DR3 and DRw6 (donor WK). In one donor (donor WC) antigen HLA-DR9, present on lymphocytes, could not be detected on RPE. This can be explained by poor expression of the antigen or by poor reactivity of the sera used. False assignment of antigen A3 (donor WC), DR7 (donors WA, HH) and Cw8 instead of Cw2 (donor WA) cannot be explained by cross reactivity. However, it might be a result of poor viability of lymphocytes or RPE. No explanation can be provided for donor HW, where an antigen DR3, absent on lymphocytes, was assigned on RPE. Likewise, explanations are lacking for donors HH and PL, in which HLA-B35 on lymphocytes was defined as B40, and B12 as B37, respectively. The method described provides an excellent possibility for post mortem HLA (Class I and Class II) typing of corneal donors with the help of RPE cells. Because of the high concordance between HLA phenotypes (96.5%) obtained from lymphocytes and RPE, we believe that RPE may substitute for lymphocytes in the conventional NIH cytotoxicity assay. Thus, every donor with corneas suitable for transplantation can be typed, regardless of how many hours post mortem eyes are retrieved (up to 42 h in our donors). This should significantly increase the proportion of corneas with known HLA type.

Because corneal transplants can be preserved for 4 wk in organ culture, there is ample time for the RPE cell culture, optimal induction of HLA Class I and Class II antigens, and typing. In the case of ambiguous serologic results, there is even time for retyping, as RPE cells grow rapidly. In addition, RPE cell cultures can be stored frozen, providing a source of donor cells that can be used as target cells for cytotoxic T lymphocytes in experiments that could help elucidate donor-recipient immunologic interactions.

The patient who needs a matched corneal transplant would benefit from a shorter waiting time for his or her matched graft, keratoplasty “at random” could become unnecessary, and long-term graft survival may be improved.

**Key words**: human retinal pigment epithelium, serologic HLA typing, induction of HLA antigens, IFN-γ, TNF-α, cytokines, corneal transplantation, high risk recipient, organ culture, cell culture

**Acknowledgments**

The authors are indebted to Jadwiga Bezcak for her excellent technical assistance, to Dr. Adolf for his gift of IFN-γ and TNF-α, and to Dr. R. Doyle Stulting for his helpful discussion.

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