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Expression of MHC class I and II molecules by cadaver retinal pigment epithelium cells: optimization of post-mortem HLA typing

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SUMMARY

The objective of this study was to investigate the expression of MHC antigens by retinal pigment epithelium cells (RPE) after stimulation with interferon-gamma (IFN-γ) and to improve the currently practised technique of cadaver HLA typing. A concentration of 100 U/ml IFN-γ induced expression of class I molecules up to >90% 3 days after stimulation, whereas 50 U/ml were required for the expression of HLA-DR to >90%. A concentration of 750 U/ml induced 35–45% expression of HLA-DP and <25% HLA-DQ after 3 days. Cells were serologically typed using the standard lymphocytotoxicity assay 3 days after stimulation with 250 U/ml IFN-γ. Typing of class I specificities was complemented by one-dimensional isoelectric focusing (1D-IEF). We observed high concordance between the results of the RPE typing and the lymphocytotoxicity test on the same donors. Our results show complete typing of class I and II antigens post-mortem, which, in particular, enables graft matching and improvement of graft survival in recipients of organs removed many hours after death such as the cornea.

Keywords HLA typing cornea transplantation

INTRODUCTION

Organ transplantation is limited by the lack of matched donor tissue. Corneal grafts are known to enjoy a high degree of immunological privilege, primarily due to the poor vascularization of the normal recipient graft bed (Katami *et al.*, 1989). However, for presensitized patients or those with a vascularized graft bed, graft rejection remains a severe threat to the allograft, necessitating full tissue typing and matching before corneal transplantation (Faure, 1964; Maumensee, 1951; Stark *et al.*, 1972). Unlike other organs, the cornea can be explanted many hours post-mortem and can be used for allografting. HLA typing on peripheral blood lymphocytes using the standard lymphocytotoxicity test is already impossible a few hours after death since viable lymphocytes are no longer available. Baumgartner *et al.* (1989a) have reported post-mortem typing of donor retinal pigment epithelium cells (RPE) by double fluorescence.

The aim of the current study was to develop a reliable technique by which cadavers can be fully HLA typed within the shortest possible time, thus minimizing conservation of explanted cornea. Using the technique described by Baumgartner *et al.* (1989a), many HLA-A, -B and -C specificities were previously declared blank. We adopted the technique established by Flood, Gouras & Kjeldbye (1980) for the separation and cultivation of RPE cells and investigated class I and II expression after interferon-gamma (IFN-γ) stimulation. By optimizing the IFN-γ concentration and cell cultivation, we successfully typed most RPE within 14 days after explantation using the standard cytotoxicity assay and one-dimensional isoelectric focusing (1D-IEF).

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MATERIALS AND METHODS

Cell cultures

Cell cultures were RPE obtained from 24 human donors within the age range 18–85 years, 3–36·5 h post-mortem. The eyes were immediately processed under sterile conditions according to Flood *et al.* (1980), with slight modifications. Briefly, after removal of the neural retina at the optic disc the shell was washed three times with Hanks' solution (GIBCO Biocult, Paisley, UK), filled with 0·25% trypsin (GIBCO) and incubated at 37°C for 20 min. The trypsin solution was removed and the whole process repeated twice. The cell suspension was centrifuged at 1200 r/min in Hams' F-10 medium (GIBCO) supplemented with 20% fetal calf serum (FCS) (GIBCO). The medium was discarded and the RPE resuspended and cultured in fresh Hams' F-10 medium in a moist chamber at 37°C and 5% CO_2–95% air atmosphere. Cell growth was checked daily by contrast-phase light microscopy. Depending on the rate of cell growth, culture medium was replenished every 2–3 days.

RPE stimulation with IFN-γ and fluorescence-activated cell sorting (FACS) analysis
Each of seven culture flasks containing 5×10^5 cells from the same donor was stimulated by addition of 50, 100, 150, 200, 250, 500, 750 U/ml recombinant human IFN-γ (Boehringer Mannheim, Germany), respectively, on days 0, 2, 3, 4, 5 and 7. Culture medium was discarded and the cells washed with phosphate-buffered saline (PBS) before addition of 0·25% trypsin solution. The culture flasks were kept at 37°C for 5–10 min. The cell suspension was centrifuged and the cells resuspended in Hams' F-10 medium. Then 5×10^5 cells were incubated with the antibodies: the anti-DR, IOT 2a (Immunotech); the anti-DQ, SPV-L3 (Immunotech); the anti-DP, 144-AS and the anti-class I heavy chain W6/32 (ATCC, Rockville, MD) for 30 min at 4°C and then washed three times. The cells were further incubated with a goat anti-mouse IgG (Jackson Laboratories, Bar Harbor, ME) coupled to fluorescein isothiocyanate. All antibodies were diluted 1/20 before use. Cells were rewashed and cell fluorescence measured on a FACStar Plus (Becton Dickinson, Mountain View, CA).

Serological typing
The standard lymphocytotoxicity test (Terasaki & McClelland, 1964), was applied using the alloantisera distributed at the 1987 International Histocompatibility Workshop and Conference.

1D-IEF and blotting
Preparation of samples and 1D-IEF was performed according to Neefjes *et al.* (1986) and Stam *et al.* (1986). Only $2{\cdot}5 \times 10^6$ cells were required for each test. Gels were washed five times in 20-min cycles in fixing solution containing 10% acetic acid, 40% methanol and 50% distilled water. Proteins were transferred at 412 mA for 4 h on to Immobilon-P transfer membranes (Millipore, FRG) according to Towbin, Staehelin & Gordon (1979).

Incubation of the transfer membranes with antibodies
The transfer membranes were washed three times for 10 min in PBS, pH 7·2, supplemented with 1% Tween 20 and thereafter incubated with 2·5% bovine serum albumin for 45 min at room temperature with shaking. The membrane was washed again three times before further incubation with the anti-heavy chain rabbit anti-human (RaHu) serum, kindly provided by Dr Stam, Amsterdam, The Netherlands, for either 2 h or overnight. The antibody was diluted 1/1000 before use. The transfer membrane was again washed three times and further incubated in alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Research Laboratories) for 1 h at room temperature. The antibody was also diluted 1/1000. After three wash cycles, class I heavy chains were visualized by addition of substrate solution as described by Dobbe *et al.* (1988).

RESULTS

Cell growth patterns were essentially as reported previously by Flood *et al.* (1980) with a few significant differences. In our cultures ($n = 24$) cells became adherent to the bottom of the culture flask after only 2–3 days. Significant cell growth was observed between days 5 and 10. Only 10–30% of the cells remained non-adherent and non-proliferative. These cells, termed 'stationary cells', were discarded after 5 days in culture.

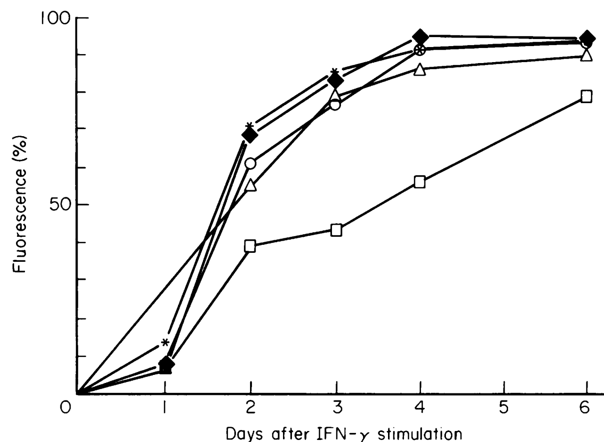

Fig. 1. Expression of class I molecules by human cadaver RPE after stimulation with IFN-γ. The anti-heavy chain monoclonal antibody W6/32 was used for the identification of class I molecules. A concentration of 100 U/ml IFN-γ was sufficient to induce maximal expression of class I molecules by > 90% cells after 3–4 days. Higher concentrations of IFN-γ did not significantly increase expression of class I molecules. ◆, 750 U/ml; *, 500 U/ml; ○, 250 U/ml; △, 100 U/ml; □, 50 U/ml.

Between 70 and 80% of cell pigment had been lost by day 12 on which most cells were serologically typed. The time lapse from donor's death to cell culture initiation significantly influenced the rate of cell growth. RPE cells which were initiated in culture more than 20 h post-mortem ($n = 11$) grew less rapidly than those initiated earlier. The mean time which elapsed between donor's death and cell culture initiation was 20·9 h. Sixteen of the 24 donors typed were both serologically and biochemically typed in less than 14 days post-mortem. Cell growth was extremely poor in only one case. The donor was a 63-year-old man and cell preparation had been completed 36·5 h post mortem.

Non-stimulated RPE express neither class I nor class II MHC molecules. RPE expressed up to 85–90% class I molecules after 3 days post-stimulation with 100 U/ml IFN-γ (Fig. 1). Higher concentrations did not increase class I expression significantly. Cells expressed only 40–50% of maximal levels of class I molecules 3 days after stimulation with 50 U/ml. In contrast, 50 U/ml IFN-γ were required to induce expression of HLA-DR molecules to > 94% whereas 750 U/ml were required for the expression of up to 35% HLA-DP and < 25% HLA-DQ after 3 days post-stimulation (Fig. 2).

Figure 3 shows the biochemical typing by 1D-IEF of a few representative donor RPE.

In six cases the lymphocytotoxicity test had been performed before death. These results were compared with those obtained on RPE post mortem (Table 1). HLA typing of donors H2, H16, H21 and H22 on lymphocytes was exactly identical to that on RPE (when results of the cytotoxicity test and 1D-IEF were put together) for A and B specificities. The cytotoxicity test on RPE of donor H5 identified HLA-A24, Bw35 and Cw4, but failed to identify HLA-A3 and -Bw61, which were visible as clear bands on the 1D-IEF blots. In contrast, the lymphocytotoxicity test had failed to identify HLA-A24. Donor H8 had been typed on lymphocytes as HLA-A24/31, B8/w55, Cw3. These specificities were clearly identifiable on the IEF blots. The cytotoxicity test

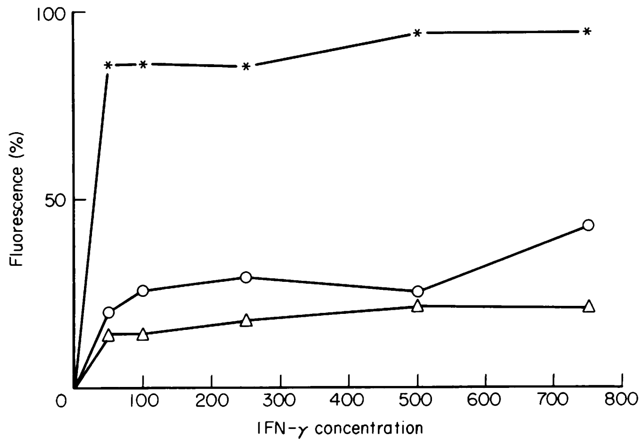

Fig. 2. Expression of MHC class II molecules by cadaver RPE. Cells were stimulated by 50, 100, 250, 500 and 750 U/ml IFN-γ. On day 3 after stimulation, expression of class II molecules was measured by FACS analysis. A concentration of 50 U/ml was required to induce expression of HLA-DR to >94%, HLA-DP to 35% and HLA-DQ to <25%. Significant increase in expression of HLA-DP was observed after stimulation with 750 U/ml, but not that of HLA-DR and HLA-DQ. *, HLA-DR; o, HLA-DP; △, HLA-DQ.

Table 1. Comparison of the RPE and lymphocytes typing

Donor	Method	HLA-A	HLA-B	HLA-C	HLA-DR
H2	RPEc	1/—	7/8		1/4
	IEF	1/24	7/8		
	Lc	1/24	7/8		1/4
H5	RPEc	24/—	35/–	w4	
	IEF	3/24	35/w61		
	Lc	3/	35/w61	w2/4	1/w11
H8	RPEc	24/–	8/w55	w3	
	IEF	24/31	8/w55		
	Lc	24/31	8/w55	w3	3/w6
H16	RPEc	2/3	w61/w62	w3	6/8
	IEF	2/3	w61/w62		
	Lc	2/3	w61/62	w2/w3	
H21	RPE	2/31	44/51		1/2
	IEF	2/31	44/51		
	Lc	2/31	44/51		1/2
H22	RPE	2/30	38/51		
	IEF	2/30	38/51		
	Lc	2/30	38/51		1/w6

RPEc, cytotoxicity test on RPE; IEF, one-dimensional isoelectric focusing; Lc, lymphocytotoxicity assay.

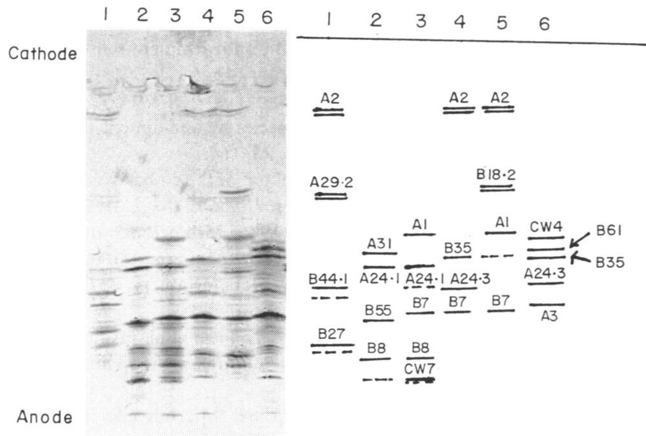

Fig. 3. One-dimensional isoelectric focusing analysis of RPE. After 1D-IEF, class I peptides were visualized by immunoblotting using the anti-class I heavy chain, RaHu serum. On the left half, the blot of six donor RPE is shown. The bands, each representing a class I specificity, are schematically drawn on the right half of the figure. Undefined specificities, likely to be HLA-C specificities, are represented by broken lines.

on the RPE had not identified A31. Some C-locus specificities have not yet been clearly defined by 1D-IEF. Undefined bands likely to be C specificities but also possibly non-polymorphic class I peptides are marked with dotted lines on Fig. 3.

DISCUSSION

Data obtained in the current study clearly present optimization of culture conditions for the promotion of rapid cell growth thus enabling early HLA typing of RPE cells. Cultivation of RPE has been in progress since the early 1920s (Fischer, 1922; Smith, 1920); however, the first comprehensive *in vitro* study of the growth patterns and ultrastructure of human RPE was reported by Flood *et al.* (1980). In contrast to their findings, we observed cell adherence to the bottom of the culture flask as early as day 2 after cell culture initiation. At this stage, cells had begun to shed their pigment. By day 5–7, mitotic division became apparent as depicted by increased cell numbers. The lag phase in our cultures was only a few days as opposed to the 2 weeks observed by Flood *et al.* (1980). Our findings agree with those of Mannagh, Arya & Irvine (1973). In our cultures, cell growth did not appear to be dependent on donor's age but rather on the time lapse between donor's death and cell culture initiation. Cell growth was extremely poor in one case, where cell culture initiation had been performed 36·5 h post-mortem. The reason why our cell cultures grew rapidly soon after culture initiation without a long time lag was probably due to the fact that we only allowed a 20-min incubation time of trypsin solution with the RPE. Trypsin might reduce the viability of cells, such that the rate of proliferation is poor. Others (Flood *et al.*, 1980) allowed 1 h incubation before aspiration of the cell suspension. In addition, culture medium was added to the cell suspension containing the trypsin solution. We immediately inactivated trypsin by addition of 20% FCS in Hams' F-10 culture medium. The cell suspension was centrifuged and the solution discarded. Cells were cultivated in fresh medium.

Previous studies by Liversidge, Sewell & Forrester (1988) on *in vitro* expression of class II molecules by RPE cells show that 1000 U/ml IFN-γ induce maximal expression after 8 days. A concentration of 500 U/ml induced maximal expression of DQ by 80% and that of DR by 70% of the RPE cells. We studied the expression of both class I and class II molecules by FACS analysis. By measuring the direct fluorescence of cells, which is

more sensitive than cell staining (Liversidge *et al.*, 1988), we were able to show that lower concentrations of IFN-γ are sufficient for cell stimulation and that class II molecules of different gene loci follow different kinetics in their response to exogenously added recombinant IFN-γ.

Recently, Baumgartner, Mayr & Grabner (1989b) reported serological typing of RPE, using a double fluorescence technique after cell stimulation with 750 U/ml IFN-γ. Initially, we applied this concentration on five donor cell cultures and used the cytotoxicity test to type the cells. Our results were extremely poor: four of the donor cell cultures had at least three or four possible HLA-A or three or four HLA-B specificities. The FACS results allowed us to reduce the IFN-γ concentration and to perform typing earlier, i.e. on the third day in contrast to 5–7 days as described by Baumgartner *et al.* (1989a). Although expression of HLA-DP and -DQ molecules was low at 250 U/ml, antigen expression was sufficient to allow satisfactory serological typing. These two variations have resulted in satisfactory serological findings. Since the cytotoxicity assay is in wide application in many laboratories, our modifications should provide useful tools for successful cadaver typing.

We obtained high correlation of the RPE and lymphocytotoxicity typing results. However, there were cases where the serological typing on RPE was still difficult, i.e. more than a single specificity was positive for one gene locus. In such cases, we introduced the well-established 1D-IEF. Since several HLA specificities have the same isoelectric point, it is not feasible to apply the 1D-IEF independent of the serology for HLA typing. This method is, however, very useful for confirmation of unclear serological results, such that both methods used together complement each other well. In contrast to the findings of Baumgartner *et al.* (1989a) we were able to type fully the RPE and eliminate possible blanks which are in fact biochemically easily identifiable specificities. Since the 1D-IEF has not been established for class II molecules, we have restricted it to class I specificities. We are in the process of establishing the restriction fragment length polymorphisms technique and hope to apply it to class II typing soon.

In this study we show that complete HLA typing of cadaver RPE is possible within 12–14 days post-mortem. This relatively short duration reduces the conservation of corneas, thus allowing early grafting. Full RPE typing as presented here is of great advantage for establishing cornea graft and possibly also heart valve graft banks. By enabling conservation of fully typed organs, transplanting of matched grafts would further reduce graft rejection in both sensitized and now-sensitized patients.

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