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Loss of corneal Langerhans cells during storage in organ culture medium, Optisol and McCarey–Kaufman medium

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Abstract

Purpose **This study compares the influence of different corneal preservation media on HLA-DR positive corneal Langerhans cells (LCs).**
Methods **Using fluorescence-associated immunohistochemistry, corneal sections were stained for HLA-DR antigens after preserving corneal-scleral discs in three different storage media: organ culture medium, Optisol and McCarey–Kaufman medium. HLA-DR positive LCs were present in corneal epithelium and upper stroma of fresh corneas.**
Results **A storage period of even 3 days had a significant influence on the number of HLA-DR positive corneal LCs. The number of LCs decreased at the limbus from 15.3 ± 4.1 LCs/0.25 mm^2 to 11.8 ± 1.2 LCs/0.25 mm^2 (p<0.01) during preservation in McCarey–Kaufman medium, to 11.2 ± 1.9 LCs/0.25mm^2 (p<0.01) during preservation in organ culture medium and to 12.7 ± 3.4 LCs/0.25 mm^2 (p<0.01) during preservation in Optisol. A greater loss was detected after 7 days and we found a cell number of 1.6 ± 1.1 LCs/0.25 mm^2 (p<0.001) after storage in organ culture medium and of 1.4 ± 1.5 LCs/0.25 mm^2 (p<0.001) after storage in Optisol. The donor tissues entirely lacked HLA-DR positive LCs, regardless of the preservation medium used, when stored for up to 14 days.**
Conclusion **These results demonstrate that loss of HLA-DR antigens is mainly related to storage period and is independent of the type of preservation medium and preservation temperature.**

Key words HLA-DR, Langerhans cells, McCarey–Kaufman medium, Optisol, Organ culture medium, Passenger leucocytes

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Several suitable corneal storage media have become available in the last 20 years. McCarey–Kaufman (MK) medium allows a storage time up to 4 days,[1] Optisol (Chiron Ophthalmics, USA) up to 14 days[2] and organ culture medium up to 48 days.[3,4]

Langerhans cells (LCs) are antigen presenting cells expressing HLA-DR antigens.[5] HLA-DR is known to play a key role in transplantation immunology[6] and LCs are the only cells in the cornea expressing HLA-DR. The HLA-DR antigen is, together with ATPase staining, the main marker for detection of corneal LCs.[6,7]

Corneal storage at 37 °C is known to have an influence on the number of corneal LCs. Pels and Van der Gaag[7] documented loss of corneal LCs in the central cornea during a preservation period of up to 4 days in organ culture medium and complete loss of LCs when stored for up to 7 days. Pepose *et al.*[8] showed that corneal storage at a temperature of 4 °C had no significant influence on the decrease in LCs, but these experiments were limited to a preservation time of up to 3 days.

The presence of donor LCs is thought to play a role in corneal immune rejection.[9–11] Our own experience has shown that transplantation of corneal buttons with a preservation time of 9.8 ± 4.7 days gives better results than transplantation using tissues with a storage time of 1.9 ± 1.4 days.[12] In this study we investigated whether there is any difference in the distribution of corneal LCs after preservation in three different storage media. Since Optisol and MK medium require a storage temperature of 4 °C and preservation in organ culture medium has to be performed at 31–37 °C, we were also interested to discover whether the storage temperature had an influence on the number of LCs.[1–4]

Materials and methods

Tissue samples

Human donor eyes were used as a source of corneal tissue. Human eyes were obtained from

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the Department of Pathology, Karl Franzens University, Graz, and prepared for preservation at the University Eye Clinic, Department of Experimental Ophthalmology. Full details of corneal preparation have been published previously by Böhnke.[13]

Eight fresh corneal-scleral discs were snap-frozen with 2-methylbutane in liquid nitrogen and stored at −70 °C until staining. Twenty-four corneal-scleral discs were put into organ culture medium (50 ml per disc) and stored at 37 °C. A further 24 discs were put into Optisol (20 ml per disc) and stored at 4 °C. Since MK medium allows adequate preservation for 4 days,[3,10] eight corneal-scleral discs were put into MK medium (20 ml per disc) and preserved at 4 °C. Eight corneas stored in Optisol, eight in organ culture medium and eight in MK medium were removed after 3 days. After a preservation time of 7 days a further 16 discs stored in Optisol and organ culture medium (8 in Optisol and 8 in organ culture medium) were removed. The remaining 16 corneas (8 in Optisol and 8 in organ culture medium) were examined after 14 days of storage. After removal from the medium, the corneas were immediately snap-frozen with 2-methylbutane in liquid nitrogen and stored at −70 °C until immunofluorescence staining was performed. All tissues were sectioned at 6 μm using a Reichert Jung cryostat (Leica-Reichert) and stained on the same day.

Immunofluorescence staining

The 6 μm thick sections were air-dried, fixed in acetone for 10 min and rinsed with phosphate-buffered saline (PBS), pH 7.4. Sections were then incubated in a dark moist chamber at room temperature with a fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human HLA-DR antibody (Becton-Dickinson, diluted 1/30 in PBS) for 30 min. They were then washed three times in PBS for 10 min each, rinsed in distilled water, air-dried and finally mounted with Mowiol 4-88 (Hoechst). Sections incubated with FITC-conjugated rabbit anti-mouse antibodies (Dako, diluted 1/30 in PBS) served as controls and were negative in all cases. The sections were scored on the same day using a Zeiss Axioplan fluorescence microscope.[9]

Counting method

Three non-serial sections of each cornea were examined and the mean number of LCs recorded. Counting was performed under a Zeiss Axioplan fluorescence microscope by two independent observers. LCs were counted per 0.25 mm^2.

The LCs of the corneal sections were counted in three different areas: (1) at the limbus, (2) in the peripheral cornea (the area stretching 2.0 mm from the limbus into the cornea) and (3) in the central cornea.

Statistics

Statistical analysis of the results was performed using the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis test. A p-value <0.05 was considered significant.

Results

Fresh corneas

Like other authors we observed high concentrations of HLA-DR positive LCs at the limbus and in the peripheral cornea of fresh corneas.[6,7] HLA-DR antigens could be detected at the basal layer of the corneal epithelium and especially in the upper stroma. Cell numbers were 15.3 ± 4.1 LCs/0.25 mm^2 at the limbus, 7.7 ± 1.3 LCs/0.25 mm^2 in the peripheral cornea and 3.2 ± 1 LCs/0.25 mm^2 in the central cornea (Fig. 1). The HLA-DR positive cells of the central cornea were limited to the epithelium or were found just beneath Bowman's membrane.

Three days storage time

MK medium

Corneas stored in MK medium for up to 3 days showed a small but significant decrease in the number of LCs at the limbus and no significant cell loss in the peripheral and central cornea, compared with fresh corneas. The cell number decreased at the limbus to 11.8 ± 1.2 LCs/0.25 mm^2 ($p<0.01$), in the peripheral cornea to 7 ± 1.3 LCs/0.25 mm^2 ($p<0.08$) and in the central cornea to 3.6 ± 0.4 LCs/0.25 mm^2 ($p<0.8$).

Organ culture medium

The number of corneal LCs after a storage time of up to 3 days in organ culture medium was 11.2 ± 1.9 LCs/0.25 mm^2 at the limbus, 4.1 ± 1 LCs/0.25 mm^2 in the peripheral cornea and 2 ± 0.6 LCs/0.25 mm^2 in the corneal cornea. This cell loss was significant at the limbus ($p<0.01$) and in the peripheral cornea ($p<0.01$), but not in the central cornea ($p<0.85$) compared with fresh corneas.

Optisol

Preservation time of up to 3 days in Optisol had a similar influence on the number of corneal LCs as did storage for the same time in MK medium or organ culture medium and showed a small but significant effect on the distribution of LCs compared with fresh corneas. The cell numbers were 12.7 ± 3.4 LCs/0.25 mm^2 at the limbus, 6.8 ± 0.4 LCs/0.25 mm^2 in the peripheral cornea and 4.5 ± 1.1 LCs/0.25 mm^2 in the central cornea. This reduction in LCs was, compared with fresh corneas, significantly lower at the limbus ($p<0.01$) but not in the peripheral ($p<0.08$) and central ($p<0.9$) cornea.

We also evaluated whether the reduction in corneal LCs after 3 days was greater in any one of the three storage media, but no significant difference could be found in any part of the cornea (limbus: $p<0.7$; peripheral cornea: $p<0.8$; central cornea: $p<0.9$).

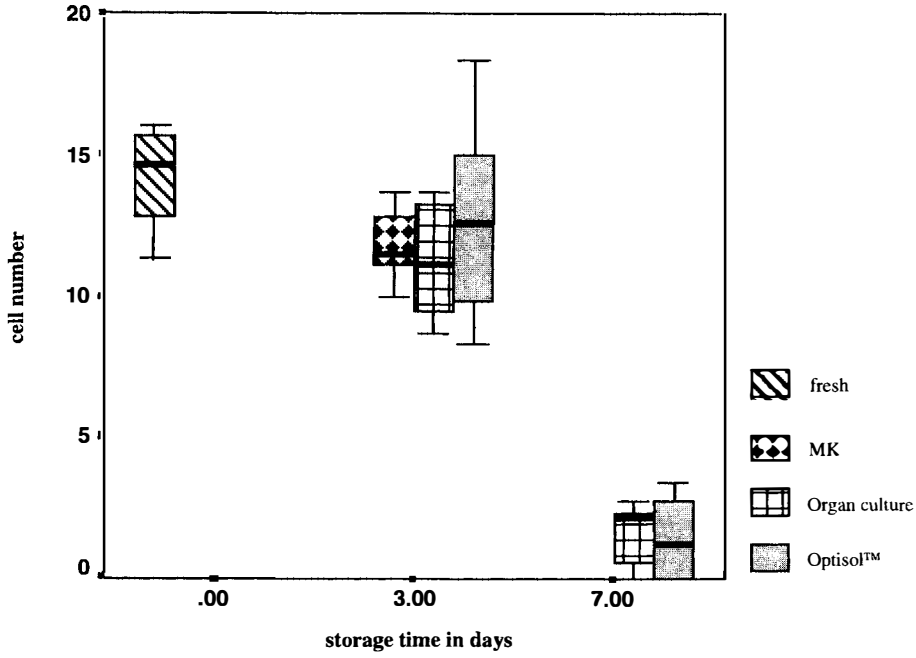

Fig. 1. *Number of limbal Langerhans cells (LCs) during storage. Cell numbers of LCs at the limbus per 0.25 mm² in fresh corneas and in corneas after storage in McCarey-Kaufman medium (MK), organ culture medium and Optisol storage medium are given.*

Seven days storage time

A storage time of 7 days led to a significant reduction in LCs in all parts of the corneal tissue. Since MK medium only allows a storage period up to 96 h, we did not store corneal tissues in this medium for 7 or 14 days.

Organ culture medium

Corneal tissues preserved in organ culture medium for up to 7 days showed a cell number of 1.6 ± 1.1 LCs/0.25 mm² at the limbus, 0.4 ± 0.2 LCs/0.25 mm² in the peripheral cornea and 0.2 ± 0.2 LCs/0.25 mm² in the central cornea. This loss of LCs was significant compared with fresh corneas ($p<0.001$) and with corneas stored for up to 3 days ($p<0.001$).

Optisol

The cell numbers of corneal LCs in tissue preserved in Optisol were very similar to those stored in organ culture medium for 7 days. The limbus had a cell number of 1.4 ± 1.5 LCs/0.25 mm², the peripheral cornea was 0.6 ± 0.2 LCs/0.25 mm² and the central cornea lacked expression of HLA-DR positive LCs in all cases. Compared with fresh corneas or corneas stored for up to 3 days a significant difference was found ($p<0.001$ in both cases).

Comparison of cell number in tissues stored for up to 7 days in organ culture medium with those preserved for up to 7 days in Optisol showed no significant difference at the limbus ($p<0.9$) or peripheral cornea ($p<0.6$). But in contrast to grafts preserved in organ culture medium, those stored in Optisol were free of LCs in the central cornea.

Fourteen days storage time

Staining of the tissues stored for 14 days demonstrated complete loss of HLA-DR antigens in all corneal buttons.

Discussion

Part of this work confirms the findings of earlier studies that corneal preservation in organ culture leads to loss of HLA-DR positive LCs.[6,7] This study shows that a significant loss of LCs bearing HLA-DR antigens does not depend on the type of storage medium or storage temperature, but on the preservation period only. The studies of Pels and Van der Gaag[7] revealed that loss of HLA-DR antigens is not based on loss of antigen presentation through downregulation, but loss of the HLA-DR expressing LCs.[7] Our experience supports their findings, showing loss of HLA-DR positive cells in stored cornea and demonstrating those cells in the storage medium by fluorescence-activated cell sorting.[14] Based on these results, the absence of HLA-DR antigens gives us a sufficient marker for the absence of LCs.

HLA-DR positive LCs are found in the epithelium and upper stroma. Their concentration is highest at the limbus and peripheral cornea and diminishes towards the centre, but they are also found in the central cornea in the epithelium and just beneath Bowman's layer.

Like the investigations of Pels and Van der Gaag[7] this study also demonstrates that storage of corneal buttons for 3 days has an influence on the reduction of HLA-DR

positive LCs. These results also confirm the studies of Holland *et al.*[6] showing that a storage time of 7 days reduces most of the corneal LCs, but are partly in contrast with those of Pepose *et al.*,[8] who found that a storage time of 3 days had no effect on the distribution of HLA-DR antigens. But in contrast to other studies this investigation evaluated loss of corneal LCs during a preservation time of up to 14 days in three different storage media at two different storage temperatures (as storage in MK medium and Optisol requires a temperature of 4 °C and preservation in organ culture medium demands a temperature of 31–37 °C)[2,15] and the decrease in LCs was additionally explored using a statistical analysis system. Since storage in MK medium is possible for up to 4 days, we did not examine the influence of a longer storage period in MK medium on the loss of HLA-DR positive LCs.

Even a storage time of 3 days in any of the three different preservation media had a small but significant influence on the number of corneal LCs. But we could not find any significant difference between the media regarding cell loss. Cell loss was greater between 3 and 7 days of storage than during the first 3 days of preservation time. After a storage time of 7 days only few LCs could be found in the tissue (1.4 ± 1.5 to 0.2 ± 0.2 LCs/0.25 mm^2). After 7 days both preservation media had led to the same quantitative loss of LCs (Fig. 1) and corneas stored for up to 14 days lack expression of LCs in any part of the tissue regardless of the preservation medium. This phenomenon is on the one hand the consequence of loss of epithelial cell layers with the epithelial LCs, and on the other hand because the stromal LCs seem to leave the tissue. The reason for this is unknown, but changes in the activity of epidermal LCs could be observed during storage in tissue culture medium.[16] Further investigations are necessary to explain the stromal loss of corneal LCs during preservation.

LCs are the only cells of the normal cornea expressing HLA-DR antigens.[7] This antigen appears to play a key role in the generation of cytotoxic T-cells, which are the primary destructive cells in the allograft rejection reaction. The enhanced immunogenicity of the allograft was demonstrated by significant elevation of the cytotoxic T-lymphocyte responses.[6,9] It appears from studies that acute rejection is associated with the presence of donor LCs in the graft and that strategies to reduce or eliminate donor LCs by UV-B irradiation or organ culture storage prevent or at least prolong graft survival.[9–11] Loss of passenger leucocytes or reduced antigenicity has been proposed as a possible mechanism.[17,18] In contrast to eliminating LCs from donor tissues by storage in preservation medium, elimination by induction of low-dose UVB irradiation leads to ultrastructural abnormalities.[19]

Clinical studies of Moll *et al.*[1] did not show significant differences in the rejection rate between patients receiving organ-cultured corneas and donor tissues stored in MK medium. Völker-Dieben *et al.*[20] found better results when using organ-cultured corneas compared with corneas stored in MK medium or a humid chamber. However, neither study documented the donor tissue storage time. Our clinical experience with organ-cultured corneas demonstrated a significantly lower rejection rate using tissues with a prolonged preservation time (mean 9.8 ± 4.7 days) compared with a short preservation time (mean 1.9 ± 1.4 days).[12] Our findings are confirmed by unpublished studies of Kamiya *et al.*[21]

Since *in vitro* studies have documented the importance of HLA-DR antigens for transplantation immunology[9–11,17,18] and clinical studies have shown that grafting of transplant stored either for up to 48 days in organ culture or for up to 14 days in Optisol is possible,[3,4,22] preference should be given to donor tissues stored for up to 14 days independent of the type of preservation medium or storage temperature, based on the fact that corneal preservation is a safe and effective way to eliminate donor HLA-DR positive Langerhans cells.

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