DO TRANSPLANTED CORNEAL LIMBAL STEM CELLS SURVIVE *IN VIVO* LONG TERM? POSSIBLE TECHNIQUES TO DETECT DONOR CELL SURVIVAL BY POLYMERASE CHAIN REACTION WITH THE AMELOGENIN GENE AND Y-SPECIFIC PROBES

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SUMMARY

Purpose: **To investigate donor cell survival following corneal limbal stem cell grafting, which is based on the corneal stem cell model.**

Methods: **We describe the use of the amelogenin gene probe with the polymerase chain reaction (PCR) to detect surviving donor cells and report preliminary studies using Y-specific DNA probes.**

Results and conclusions: **DNA polymorphisms have a detection limit of 10%. The SRY 'Y-specific' probe has a theoretical detection limit of 1 cell in 10 000. The techniques were applied to investigate survival of male donor cells in an aniridic female patient $2\frac{1}{2}$ years following limbal stem cell grafts. We speculate that low levels of donor-derived cells may still be present. We discuss the advantages and disadvantages of the two approaches, which may have future clinical and experimental application.**

The concept of corneal stem cells provides the rationale for corneal stem cell grafts in conditions of stem cell dysfunction. Corneal stem cell deficiency, malfunction or absence causes loss of proliferative capacity of the corneal epithelium and results in recognised clinical features.[1]

Limbal insufficiency may be primary, as in aniridia, but is most often secondary. Acquired limbal insufficiency follows chemical or thermal injury, contact lens wear or limbal surgery, all of which cause limbal damage and ischaemia, the extent of which determines the prognosis.[2–4]

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The place of corneal limbal stem cell autografting for unilateral limbal insufficiency is well recognised.[5,6] In bilateral limbal insufficiency allografting has been shown to be feasible.[7,8] The survival of donor cells is presumed by the stabilisation of the ocular surface and regression of the features of limbal insufficiency. What is the fate of transplanted stem cells? By definition stem cells either remain as stem cells or differentiate into transient amplifying cells (TACs) and subsequently post-mitotic corneal epithelial cells with different marker characteristics. If transplanted cells retained markers whatever their state of differentiation, it would become possible to determine survival of the transplanted cell population. Medium-term survival of donor cells has been demonstrated by the use of DNA polymorphisms and the polymerase chain reaction (PCR) by Williams *et al.*[9] They showed the presence of donor cells in the central cornea of a recipient 12 weeks after limbal stem cell grafts. Donor cells were therefore detected following a period of delay, were present at low levels and their presence was transient. No detectable levels of donor cells were subsequently found at 20 weeks after grafting.

A unique clinical opportunity prompted the use of cell sex characteristics to assess stem cell viability of transplanted cells *in vivo*. A 48-year-old aniridic woman received a corneal stem cell graft from her non-aniridic but histocompatibly matched son. This study sought to detect the presence of surviving male cells by using the amelogenin gene probe with PCR, this is equivalent to the use of specific DNA polymorphisms. Preliminary studies were also carried out using Y-specific DNA probes to detect gene products from the Y chromosome to see whether this

Table I. Summary of results for the control dilution series and corneal epithelial samples using the amelogenin probe and the SRY probe

Sample	Cell type	Amelogenin probe	Y-specific SRY (39 cycles)
Blood-derived pure DNA control samples			
Pure ♀/water	♀	+ (214 base pairs)	−
Pure ♂	♂	+ (220 base pairs)	+
10:1	♀:♂	+	+
100:1	♀:♂	−	+
1000:1	♀:♂	−	+
10 000:1	♀:♂	−	+
Control corneal epithelial samples			
A	♂	+	+
B	♀	+	± (<0.1%)
C	♂	+	+
D	♂	+	+
E	♀	+	± (<0.1%)
F	♀	+	± (<0.1%)
G	♀	+	−
H	♂	+	+
I	♀	+	± (<0.1%)
J	♂	(+)	+
K	♂	+	+
L	♂	−	−
M	♂	+	+
N	♂	+	+
O	♂	+	+
P	♂	+	+
Q	♂	+	+
R	♂	(+)	+
S	♀	+	−
T	♀	+	± (<0.1%)
	13♂, 7♀	12♂, 7♀	

M, male; F, female.
−, not detected; +, detected to significant peak height; (+), confirmed on repeat masked run; (0.1%), equivalent detection level.

too could allow detection of donor cells or cells derived from them.

METHODS

Twenty control samples of corneal epithelium were harvested from donor and host material of patients undergoing routine penetrating keratoplasty. Under aseptic conditions samples of approximately 1–3 mm diameter were placed in sterile normal saline solution and sealed in micro test tubes. Samples were spun down to produce a concentrated pellet and stored for use in all experiments.

Specific DNA probes were used with PCR to look for male-derived DNA. These were:

1. The amelogenin gene probe, which detects this gene coding for dentine production with a gene product size in male cells different from that in female cells. (For PCR conditions see Appendix.)
2. The SRY probe, which detects the testis-determining gene sequence on the Y chromosome. (For PCR conditions see Appendix.)
3. The DYZ probe, which detects a multiply repeating sequence on the Y chromosome. This was used initially but did not have the sensitivity or specificity of the SRY probe. (For PCR conditions see Appendix.)

Masked testing of the corneal epithelial samples was carried out using the amelogenin probe without prior knowledge of the patient's sex.

Reconstruction experiments were carried out for all three probes using pure blood-derived DNA samples to produce dilutions of male cells within female cell samples to determine the detection limit. Following reconstruction experiments the DNA probes were tested against the control corneal epithelial samples.

All samples were analysed using the Genescan system (model 672, Applied Biosystems), which allows analysis of multiple samples in an equivalent manner to electrophoretic gels with tight control on the conditions and duration of PCR and run sequences.

RESULTS

The results are summarised in Table I. All test samples, however small, showed detectable levels of DNA. All 19 samples that amplified were correctly sexed using the amelogenin probe, giving 12 male and 7 female control samples.

Using the amelogenin probe the detection limit was found to be 10%, with a female peak at 214 bases and male peak at 220 bases (Fig. 1). From the reconstruction experiments it was possible using the SRY probe to reach a theoretical detection limit of 0.01% (Fig. 2). This is equivalent to 1 male cell in 10 000 female cells. The sensitivity of the DYZ probe was limited by non-specific amplification products and so was not used further.

With the SRY probe all 19 amplifying test corneal epithelial samples were correctly sexed whatever the size of sample. However, very low levels (0.1–1%) of Y signal were detected in some of the female control samples.

The technique described was then applied in the following specific clinical situation.

Case Report

A 48-year-old aniridic woman with chronic features of limbal stem cell deficiency in both eyes underwent corneal limbal stem cell grafts in her right eye from her non-aniridic but histocompatibly matched son. (Both mother and son were blood group B Rhesus +ve and shared the following HLA antigens: A2, B12(B44), BW4, BW6 and DR6. There were only three mismatched loci of those tested.) The aim of the procedure was to try to stabilise her right ocular surface and reduce the frequency of recurrent epithelial breakdown.

The patient underwent bilateral intracapsular cataract surgery for congenital cataracts in 1973 and 1974 and used aphakic spectacles. A short period of contact lens wear was abandoned because of corneal vascularisation. Clinical records

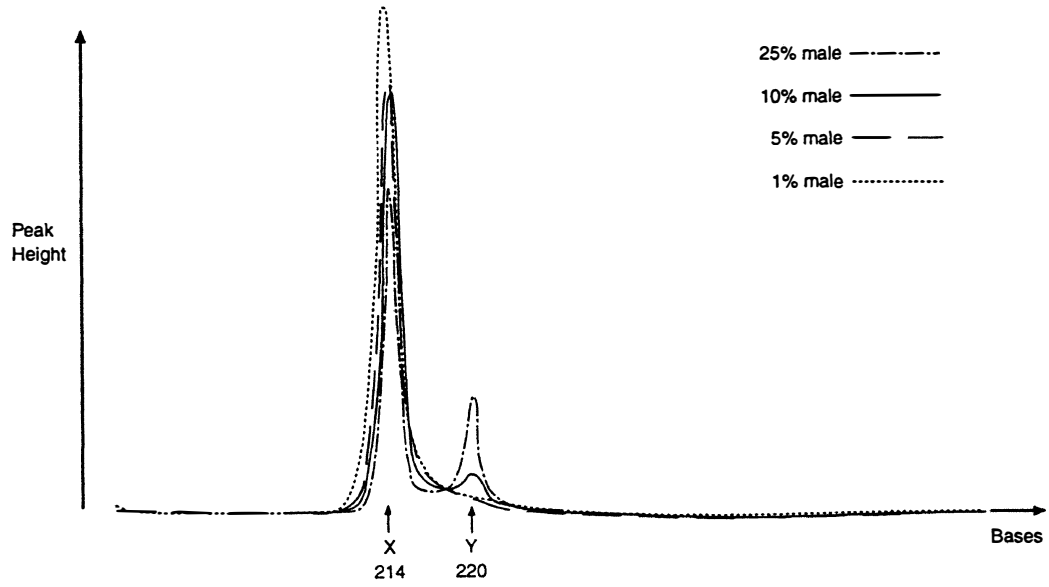


Fig. 1. *Genescan tracing showing different product peaks for samples with different dilutions of male DNA in a background of female DNA (prepared from DNA extracted from whole blood). At less than 10% male cells no separately resolvable Y peak is detectable. The detection limit with this probe is therefore 10%.*

over a period of 10 years show progression of peripheral vascularisation and stromal opacification to include the whole corneal surface in both eyes, eventually leading to bullous keratopathy. Chronic simple glaucoma was treated with timoptol 0.5% b.d. topically. A right penetrating keratoplasty was carried out in April 1987 using fresh female donor material (48 hours old). Despite settling well, by 8 months there were features suggesting progressive epithelial failure, with stromal thickening and haziness as well as increasing vascularisation. Attempts to suppress what was interpreted then as 'rejection' were unsuccessful and a repeat corneal graft was performed for graft failure 14 months after the first penetrating keratoplasty. Donor material this time was from cultured tissue 2 weeks following harvest and was again from a female donor. An early possible epithelial rejection line at 1 month resolved without aggressive topical treatment. One further probable rejection episode with endothelial keratic precipitates also resolved. However, epithelial irregularity and anterior stromal haze had developed by 1 year following grafting, which progressed to bullous keratopathy. The corneal epithelial surface thereafter remained uneven and irregular in appearance with recurrent epithelial defects despite adequate lubrication and tear supplements (Fig. 3). In an attempt to stabilise the ocular surface corneal limbal stem cell grafting was performed in the right eye.

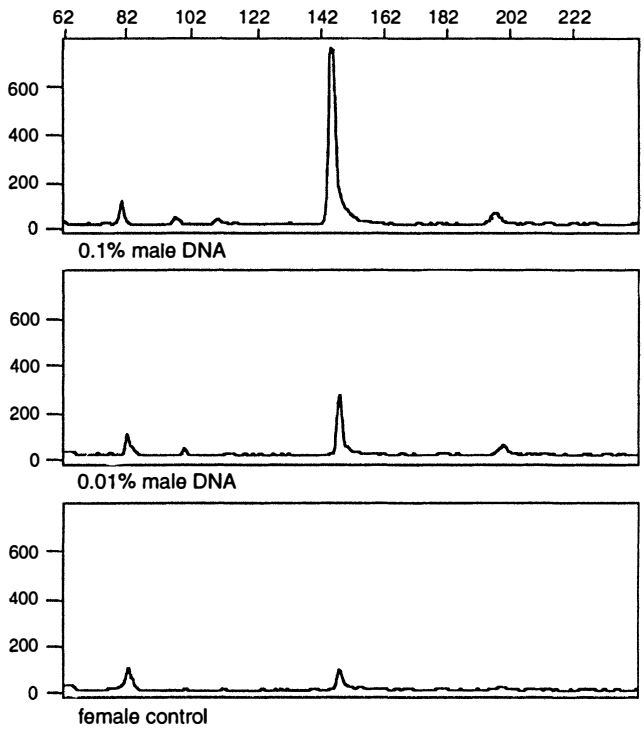
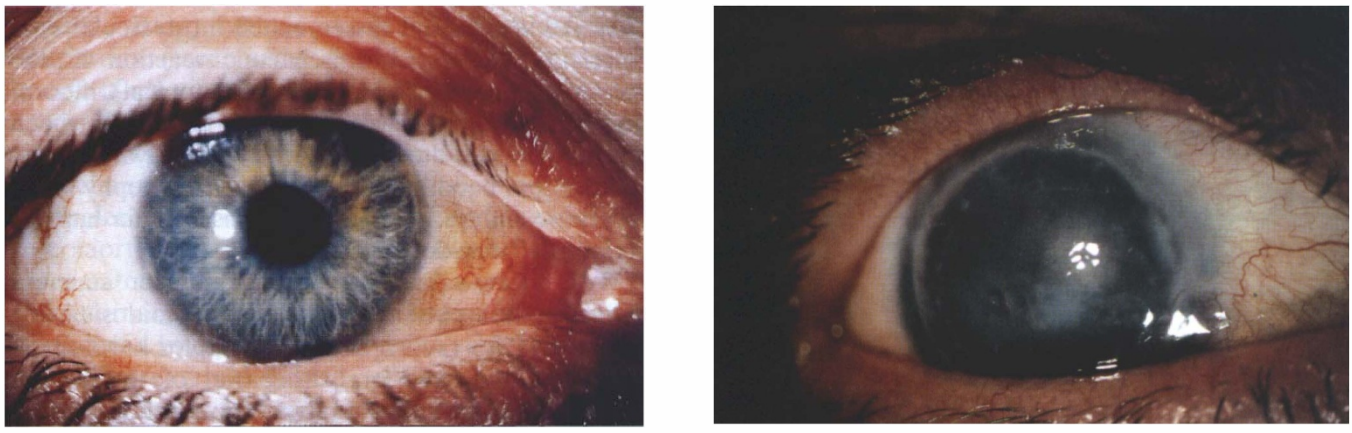


Fig. 2. *Dilution series peaks for the SRY probe showing detection of 0.01% male DNA.*

The surgical technique followed that described by Kenyon and Tseng[6] for autografts, taking two grafts each of 2 clock-hours of limbal tissue from her son that were placed in the prepared recipient sites in her right eye. Post-operative treatment was with topical steroids and antibiotic only. The transplanted allograft material appeared to take well without excessive vascularisation and no evidence of sloughing. Clinically the outcome was beneficial for her, with subjectively greater ocular comfort and brighter vision. Visual acuity improved from CF to 6/60 (with +13.00/+3.00 × 180) but, despite a reduced frequency of central epithelial defects, such defects



(a) (b)

Fig. 3. *Clinical photographs showing the appearance of both corneas at the time of obtaining corneal epithelial samples. (a) The central epithelial defect is clearly visible in the right eye. (b) The left eye has never undergone surgery.*

continued to recur. There was no corresponding definite improvement in corneal clarity or significant reduction in corneal vascularisation.

It was when the patient was reviewed with loose central epithelium adjacent to a recurrent erosion that samples were taken to see whether donor cell material could still be detected $2\frac{1}{2}$ years following her limbal stem cell grafts. Peripheral sample sites recovered within a week but the central area of recurrent defect only stabilised slowly over several weeks, as with previous episodes. In her son, donor sites re-epithelialised quickly without further problems.

Limbal Stem Cell Graft: Patient Studies. Five small test samples (approx 0.5–1.5 mm diameter) were obtained from the study patient after ethics committee approval and informed consent. One sample was taken from the loose central epithelium and four peripherally: two over the area of previous limbal grafts and two in the other quadrants (Fig. 4). Patient test samples underwent the same series of tests with the amelogenin probe and the SRY probe.

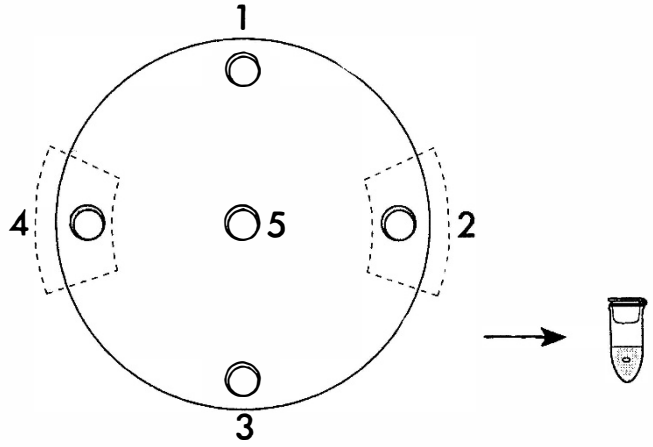


Fig. 4. *Diagram showing test sample sites in relation to the previous limbal grafts (dotted outlines). Sample 5 was loose epithelium adjacent to the persistent epithelial defect. Sample numbers correspond to those in Table II and Fig. 5.*

Test Sample Results (Table II). All patient test samples showed detectable DNA levels and all successfully amplified. Using the amelogenin probe it was not possible to detect male Y chromosome gene products to a sensitivity of 10%. With the SRY probe, all samples showed low levels of Y material to a level of 1% (Fig. 5).

DISCUSSION

In penetrating keratoplasty, transplanted corneal epithelial cells are usually replaced by host cells covering the donor corneal surface, but donor epithelial cells have been shown to survive for at least a year experimentally.[10] Late epithelial rejection is a recognised problem, also suggesting prolonged survival of transplanted epithelial basal cells. It is therefore possible that smaller numbers of cells could be surviving for longer periods.

The technique we describe uses DNA probes and PCR on small corneal epithelial samples to detect sex chromosome gene products. This technique is similar to that used for samples obtained by chorionic villous sampling (CVS).[11–13]

The first part of the study using the amelogenin gene probe supports the work of Williams *et al.*[9] using DNA polymorphisms to detect a unique donor cell 'fingerprint'. Our findings confirm that using any similar method will only allow a sensitivity

Table II. Summary of results on patient test samples using the amelogenin probe and the SRY probe

Sample	Cell type	Amelogenin probe	Y (specific) SRY (39 cycles)
1	♀:♂ (?)	–	+ (1%)
2	♀:♂ (?)	–	+ (1%)
3	♀:♂ (?)	–	+ (1%)
4	♀:♂ (?)	–	+ (1%)
5	♀:♂ (?)	–	+ (1%)

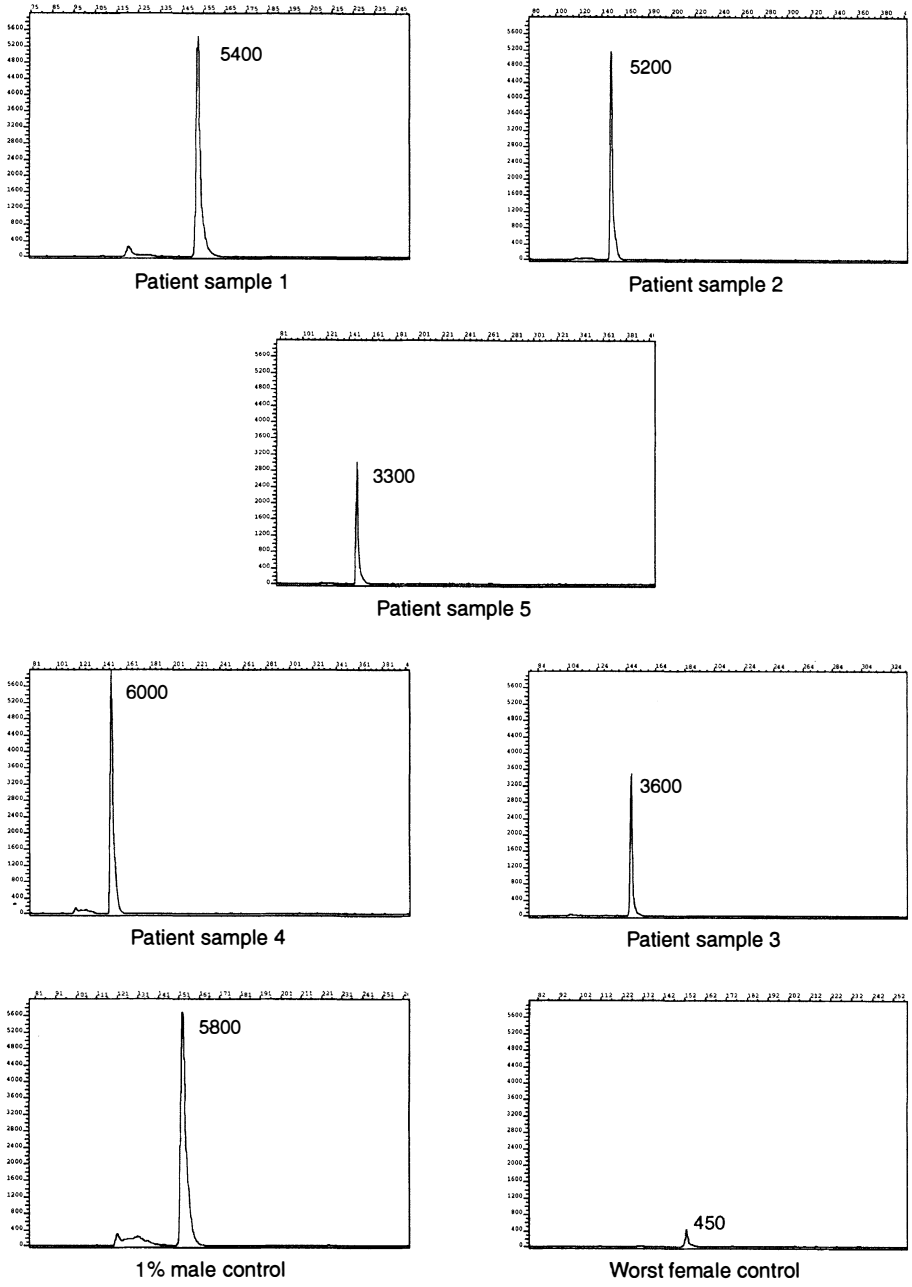


Fig. 5. *Patient sample results with the SRY probe. All test samples are shown compared with the largest peak obtained in the worst female control sample. Sample numbers correspond to those in Fig. 4. No significant difference in peak magnitude exists between samples overlying the original limbal graft sites and those not doing so.*

of 10%. This is because it is a measure of relative sensitivity. It is a highly specific technique for any particular donor–recipient combination but the choice of which DNA polymorphism to use must be repeated for every donor–recipient combination to be tested. Assuming the grafts 'took' then some cell turn-over with re-population of the corneal surface would have resulted. The studies using the amelogenin probe show that if donor cells remain present at $2\frac{1}{2}$ years after grafting it is at a level of less than 10%.

Did the limbal stem cells or any cells derived from them survive at all? If the grafts had not survived at all, with or without even a limited rejection response, some evidence of sloughing of transplanted material should have been observed very soon after surgery was carried out, and this was not the case. Even if early rejection had occurred, it is considered rare but relatively easy to control with topical steroids.[14] This begs the question of what the mechanism might be for the apparent stabilisation of the ocular surface, which has persisted, if no donor cell survival occurred.

It has been suggested that in conditions with a pathologically altered or developmentally abnormal limbal stromal micro-environment, such as aniridia,

support of limbal stem cell function may not be possible.[15] The donor graft may therefore act as a stabilising matrix for the ocular surface, stabilising the stem cell 'niche' allowing support of limbal stem cell function (whether donor or host derived) and pushing back the conjunctival–corneal cell interface which in aniridic patients can occur more centrally than at the limbus.[16,17] One further possibility is that in fact there is a relatively stable surface of mainly conjunctival-derived cells.

Comments on Preliminary Studies with Y-Specific Probes

Using the sex characteristics of donor cells to detect their survival is only applicable to mixed-sex donor–recipient combinations. The advantage of the amelogenin probe is that it can be used in any mixed sex combination. The Y-specific probes can only be used when there is a male donor and a female recipient, and theoretically allows for a far greater sensitivity with much lower levels of survival of donor cells detectable.

For the SRY probe the theoretical sensitivity reaches 0.01% in reconstruction studies. The suggestion of detectable levels of Y material in all test samples with the SRY probe is very interesting. When compared with the pure blood-derived DNA samples that show no detectable peak with either of the Y-specific probes, the results look convincing. But five of seven of the female control corneal epithelial samples showed trace but detectable levels of Y material. Although minute levels of extraneous Y material were detected, the highest levels were still 10 times lower than the positive result with the test samples using the SRY probe. We speculate that the results hint at low levels of grafted male cells $2\frac{1}{2}$ years after grafting which would not be detectable using DNA polymorphisms. This lends support for the stem cell model and the rationale behind carrying out corneal limbal stem cell grafting.

No conclusions regarding the distribution of survival donor cells relative to the grafting sites can be drawn because of the apparently low levels detected. Early on the cell density would be expected to be greatest adjacent to the graft sites. The presence of detectable donor cells in all test samples from the patient would suggest that extensive cell migration over the corneal surface is able to occur, as has been suggested by studies of models of corneal epithelial loss.[18]

Further studies intend to clarify whether these findings do represent geniune survival of low levels of donor cells or merely a tantalising but erroneous picture.

CONCLUSION

The use of the amelogenin gene appears to be a suitable method for detecting cells of one sex in an environment predominated by cells of the opposite sex using very small samples of material. However, by this means we were unable to show survival of donor cells at or above the 10% level. The use of Y-specific DNA probes could provide a means of absolute sensitivity to detect Y material against a background of female cells.

APPENDIX. PCR CONDITIONS

DNA Extraction

DNA was extracted from tissue samples by heating to 70 °C for 30 minutes with 50 μl of Instagene matrix (BioRad) followed by boiling for 10 minutes. After centrifugation for 5 minutes at 12 000 *g* the supernatant was used (5 μl in a 10 μl PCR).

PCR Amplification

Hot-start conditions were achieved using *Taq* polymerase in the presence of a monoclonal antibody (Clonetech). The presence of the antibody ensured that primer extension reactions only started when the reaction temperature was high enough to prevent non-specific reactions. Ten microlitre reactions were conducted with primers designed to amplify: (1) amelogenin, a tooth enamel gene with copies on both the X and Y chromosome, the amplification products being 214 bases and 220 bases respectively; (2) SRY, the human sex-determining gene present on the Y chromosome as a single copy.

Amplification Conditions

Amplimer	*Denature*	*Anneal*	*Extension*	*Cycles*
Amelogenin	94 °C for 1 min	61 °C for 1 min	72 °C for 1 min	39
SRY	94 °C for 1 min	63 °C for 1 min	72 °C for 2 min	39

Primer Sequences

Amelogenin Tamra-ACC-TCA-TCC-TGG-GCA-CCC-TGG-AGG-CTT-GAG-GCC-AAC-CAT-CAG
SRY 5/6-Fam-CAT-GAA-CGC-ATT-CAT-CGT-GTG-GTC-GCC-TCC-TGG-AAG-AAT-GGC-CAT-TTT

Key words: DNA polymorphisms, Y-specific DNA probes, Polymerase chain reaction (PCR), Allograft, Corneal limbal stem cells, Aniridia.

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