IN VIVO SURVIVAL OF THE ENDOTHELIUM OF FREEZE-STORED CORNEAL HOMOGRAFTS IN THE RABBIT*†

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ONE specific problem in the biological behaviour of full-thickness corneal homografts is the possibility that opacification is related to repopulation of the donor endothelium by the host endothelium. The introduction of techniques for the identification of host and donor endothelium in experimental animals, *viz.* the use of sex chromatin (Espiritu, Kara, and Tabowitz, 1961) and labelling with tritiated thymidine (Polack and Smelser, 1962), has shown that the donor endothelium survives in the majority of continuously clear long-term grafts, although occasional clear grafts have been repopulated by the host endothelium (Polack, Smelser, and Rose, 1964; Chi, Teng, and Katzin, 1965). The effect of freezing and preservation on the survival of transplanted endothelium *in vivo* is as yet undetermined, so the sex chromatin marker technique has been applied to clear grafts in a small group of rabbits which had received the transplants from animals of the opposite sex after the donor corneae had been stored in a mixture of alcohol and solid carbon dioxide at a temperature of approximately -79°C .

Materials and Methods

The corneae used for this investigation were taken from rabbits which were long-term survivors of an experiment to study the effect of preservation and freezing on the clinical and histological changes in corneal homotransplants (Mueller and Smith, 1963; Mueller, 1964). Eleven adult Dutch rabbits provided suitable material for study; all had clear grafts when the animals were killed and of these three had received fresh unfrozen grafts, while eight had received grafts which had been frozen for between 1 and 100 days.

Preparation of Donor Eyes and Surgical Technique.—The methods of cooling to, banking at, and thawing from -79°C . have been described in detail in previous papers (Smith, Ashwood-Smith, and Young, 1963; Mueller, 1964).

The anterior chambers of intact donor eyes were irrigated with a medium containing 7.5 per cent. dimethyl sulphoxide and the eyes were then suspended in large glass tubes in a medium containing 10 per cent. glycerol. The tubes were sealed and cooled at controlled rates and stored at -79°C . for various time intervals. Immediately before the grafting procedure the banked eyes were rapidly thawed out and the tubes were opened. As soon as all the ice attached to the cornea had melted, the eyes were taken out and the full thickness grafts were cut. Control eyes were irrigated with 7.5 per cent. dimethyl sulphoxide and suspended in 10 per cent. glycerol and kept at $+4^{\circ}\text{C}$. for up to 4 hours before the discs were cut.

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The 6.1 mm. full-thickness donor disc was sutured into a 6.0 mm. bed in the host by four direct interrupted virgin silk sutures to fix the graft and a continuous direct suture to seal the host-graft junction. Post-operative therapy with antibiotics and steroids was rarely necessary and the sutures were removed from each animal within the third post-operative week. The animals were examined frequently during the first six post-operative months and every second month up to 2 years after the operation.

Preparation of the Tissues for Histology and Cytology.—Immediately after the grafted animal had been killed by air embolism, the eye was removed and the cornea dissected as shown in Fig. 1. The cornea was everted over a hemispherical perspex rod which was trans-illuminated on a dissecting microscope. A modification of the technique described by Stocker, Eiring, Georgiade, and Georgiade (1958) was used for the removal of host and graft Descemet's membrane and its overlying endothelial monolayer (Fig. 1).

FIG. 1.—Diagram to show techniques for removal of Descemet's membrane and overlying endothelial monolayer.

A semicircular piece of graft membrane and a rectangular piece of host membrane were stripped from the stroma with dissecting forceps and slipped on to coverslips coated with albumen: the preparations were fixed at 45°C. over Davidson's fixative in an embedding oven. The heat fixed the membrane firmly to the coverslip which was then glued to a glass slide (Fig. 2). Sex chromatin was easily recognized in these preparations when they were

FIG. 2.—Semicircular mount of Descemet's membrane stained with buffered thionine to demonstrate endothelial nuclei. $\times 32$.

stained with buffered thionine at pH 5.7 after acid hydrolysis (Klinger and Ludwig, 1957). With this staining method a female endothelial monolayer was found to contain 40 to 60 per cent. of cell nuclei with sex chromatin (Fig. 3), while male endothelium contained less than 5 per cent. and usually 2 per cent. of cells with sex chromatin and in addition the male cells were found to have a nuclear chromatin which was more finely granular than the female cells (Fig. 4).

FIG. 3.—Female endothelial cells stained with buffered thionine after acid hydrolysis; note sex-chromatin clumps beneath the nuclear membrane. $\times 2,500$.

FIG. 4.—Male endothelial cells similarly stained; note absence of sex-chromatin and fine granular pattern of nuclear chromatin. $\times 2,500$.

When the preparations of the grafted endothelium were examined, it was easily apparent that some contained a mixed population of cells, because the nuclear sex of the cells at the edge of the graft differed from the nuclear sex of the cells at the centre. The simplest way to represent this mathematically was to take separate samples at the margin and at the centre, and in the quantitative technique adopted sample populations of 200 cells were counted at the centre and at points along the edge. The counts were made without prior knowledge of the sex of the donor or host animals and the host cornea of known sex provided a control. Good agreement was obtained between different observers when counts were made on the same preparation.

For histological study the remainder of the cornea was inverted and fixed in Davidson's solution for 24 to 48 hours. The unspoilt half of the cornea was embedded in paraffin wax and sections were cut at four levels to allow histological investigation of the graft and host tissue. The sections were stained with haematoxylin and eosin and by the periodic-acid Schiff technique.

Results

The clinical progress of the homografts in the eleven rabbits is summarized in the Table.

During the first 6 months after operation there were no serious complications. Superficial vascularization of the host and grafted cornea at the host-graft junction was associated with some oedema, but this subsided when the sutures were removed and at the end of the fourth month all the grafts were clear. Pathological changes occurred at a later date in some of the clear grafts, but the reasons for this are not readily apparent. A reasonable possibility is that the sensory nerve supply of the rabbit cornea was diminished by the keratoplasty and this insensitivity may have rendered the animal more susceptible to traumatic keratitis.

The three control animals which were observed for up to 32 months did not develop opacities. Three of the four grafts which had been stored at -79°C . and which showed cytological evidence of survival of the donor endothelium had late complications. A superficial central stromal opacity developed 7 months after operation in rabbit RCG 200: this was successfully treated by keratectomy and the graft thenceforth remained clear. The graft in rabbit RCG 205 showed a diffuse fine superficial stromal haze over the upper half of the graft, but the epithelium remained smooth and the opacity cleared during the following 6 months' observation. Rabbit RS 68 showed an opacification with fine vascularization just inside the host-graft junction which occurred 10 months after operation; although the

CLINICAL, CYTOLOGICAL, AND HISTOLOGICAL

Group	Donor Corneae	Fate of Endothelium	Animal No.	Duration of Storage at -79°C . (days)	Duration of Implantation (mths)	Clinical Details
1	Unfrozen	Survived	1 RCG 160	0	32	Slight leak of aqueous post-op
			2 RS 4	0	20	Fine peripheral opacities were cleared by 16 wks
			3 RS 8	0	20	Oedema until stitches removed Oedema until stitches removed
2	Frozen	Survived	4 RCG 200	1	25	Initially clear
			5 RCG 205	1	24	Developed superficial keratitis by keratectomy at 7 mths
			6 SHS 6	3	14	Diffuse superficial stromal haze oped at 8 months, but later cleared
			7 RS 68	45	17	Peripheral crescent developed Centre remained clear Graft contracted Persistent circumferential opacity at host-graft junction
3	Frozen	Completely Repopulated	8 RS 7	11	20	Oedema until stitches removed
			9 RS 59	38	16	Remained clear
			10 RCG 230	40	22	Developed persistent crescentic at margin of graft Centre clear
			11 RCG 254	100	21	Developed small crescentic peripheral scar Centre remained clear Fine diffuse superficial opacities oped at late stage

vascularization decreased slightly, the opacity remained and both were present when the animal was killed 17 months after operation. The graft of the animal SHS 6 remained clear for 14 months, but shrank from the original size of 6.1 mm. to about 5.5 mm. diameter; the reason for this is not understood.

Three of the four grafts which had been stored at -79°C . and which showed evidence of complete repopulation by host cells developed opacities. In two rabbits (RS 59; RCG 230), the opacities were dense peripheral wedge-shaped scars which were in the stromal layers of the graft. In rabbit RCG 254, the opacification was fine and diffuse throughout the graft and apparently involved the superficial and stromal layers: the epithelium was irregular but without defects in continuity. By comparison the opacities in the repopulated grafts were more dense than those in the grafts in which the endothelium survived. In none of the eyes were there signs of lens opacity.

The results obtained by the study of sex chromatin in the endothelial monolayers are shown in the Table.

Those grafts in which no host cells were seen are described as showing "complete" survival of the donor cells. This was found in two grafts: RCG 160 which was an unfrozen graft and had been *in situ* for 32 months, and RCG 200 which had been frozen for one day and had been in the host for 25 months.

EVEN CLEAR CORNEAL HOMOGRAFTS

Per cent. Endothelial Cells containing Sex Chromatin			Survival of Donor Endothelial Cells	Histological Findings		
ost	Graft			Descemet's Membrane		Fibrous Wedge
	Edge	Centre		Loss of Continuity	Duplicated	
50	2	2	Complete	0	0	Very small
45	20	3	Partial	+	±	Small
2	17	42	Partial	0	±	None
55	1	1	Complete	+	0	None
56	50	2	Partial	0	±	None
60	26	2	Partial	0	±	Small
66	57	12	Partial	0	±	Small
2	2	2	None	0	+	Small
40	39	42	None	0	+	Small
43	39	41	None	0	+	Large
2	2	3	None	+	+	Large

When donor cells were found only in the centre of the graft and were surrounded by host cells at the periphery, the survival was described as "partial" and this was found in two control grafts, RS 4 and RS 8, and in three grafts which had been frozen for 1, 3, and 45 days respectively (RCG 205, SHS 6, and RS 68); all five grafts had been in the host animal for between 14 and 24 months.

In the third group of four animals there was no evidence of donor survival; these grafts were frozen for 11, 38, 40, and 100 days, and had been implanted for between 16 and 22 months.

Although the histological study of the opposite half of the grafted cornea cannot be directly compared with the cytological results, some interesting information was obtained with regard to the cellular changes within the grafts. Inversion and eversion of the cornea under tension during the stripping procedure caused disruption of the stroma and damage to the corneal epithelium in many of the sections, so that reliable observations could be made only on the presence or absence of granulation tissue and the pattern of the relationship between host and donor Descemet's membrane. In the seven grafts which showed survival of the donor endothelium, the stroma of the graft was free of inflammatory infiltrate and scattered lymphocytes, and occasional capillaries were seen only at the host-graft junction. Small round cell infiltration was more prominent in those grafts which showed failure of the endothelium to survive, and focal collections of inflammatory cells were seen within the host-graft junctions and were associated with capillary formation; one graft (RCG 254) showed the changes of a deep keratitis at the periphery.

An attempt has been made in the Table to express symbolically the changes observed in Descemet's membrane. Staining by the Periodic-acid Schiff technique demonstrated the edges of the graft by revealing a double layer (Fig. 9) of the membrane. In some of the grafts this double layer extended across the whole length of the graft (designated '+' in the Table, see Figs 5 to 8, opposite), while in others there was a partial fusion with a solid membrane in part of the graft (designated '±' in the Table). When the membrane was solid in its major part with minimal splitting at the periphery (Fig. 9) it was designated '0' in

FIG. 9.—Edge of graft showing complete fusion of the two Descemet's membranes. Periodic-acid Schiff. $\times 210$.

FIG. 5.—Section of a complete corneal graft, showing limits of graft. Periodic-acid Schiff. $\times 30$.

FIG. 6.—Magnified part of graft edge, showing large fibrous wedge and loss of endothelial continuity. Periodic-acid Schiff. $\times 80$.

FIG. 7.—Centre of graft shown in Fig. 5; note duplication of Descemet's membrane. Periodic-acid Schiff. $\times 186$.

FIG. 8.—Magnified part of graft edge to show a small wedge and the double layer of Descemet's membrane. Periodic-acid Schiff. $\times 80$.

the Table. Since dense peripheral scars were a clinical feature, the presence of peripheral wedges of fibrous tissue growing between two layers of Descemet's membrane was recorded in the Table as 'large' (Fig. 6) and 'small' (Fig. 8): none of the sections showed a fibrous tissue wedge growing behind the posterior layer of Descemet's membrane. In three of the grafts the continuity of Descemet's membrane and the overlying endothelial layer was broken (Fig. 6), and the presence of this loss of continuity is expressed as '+' in the Table. Anterior synechiae were not seen in this material, although occasional clumps of pigment-containing histiocytes were seen on the bare areas.

With the proviso that the cytological and histological studies were carried out on different parts of the individual corneal graft, it would seem that the most obvious histological association with donor endothelial cell survival is an absence of inflammatory infiltrate and the presence of a fused or single Descemet's membrane. Lack of endothelial continuity and the presence of peripheral wedges seem to be of less importance.

Discussion

There are now two generally accepted methods, namely autoradiography and sex-chromatin markers, for the identification of donor endothelium in experimental corneal transplants, and each has advantages and disadvantages. Although it is unlikely that the presence of an intranuclear isotope such as tritiated thymidine would *per se* cause an alteration in cellular behaviour, the labelling process necessitates the killing of an original endothelium to provide a proliferating cell population which will take up the labelled thymidine; this experimental model is, therefore, always dogged by the possibility that the labelled graft endothelium has a modified survival potential and another unknown quantity is introduced into the system. The advantage of the autoradiographic technique is that the host and donor cells may be identified at a glance in complete corneal mounts, so that the migration of host endothelium is easily recognized (Polack and others, 1964). The identification of cells by their sex-chromatin content has the advantages that the cells have not been altered biologically, and that the technique is simple and can be used in the study of human material. The disadvantage is that recognition depends upon nuclear detail and comprehensive topographical studies are time-consuming; in addition the separation of male and female populations is statistical, so that the technique is inadequate for the assessment of 'partial' repopulation.

As a means of studying the survival of fresh homograft endothelium *in vivo*, both autoradiography and sex-chromatin markers have revealed long survival. The isotope studies of Hanna and Irwin (1962) and Polack and others (1964) gave survival times of up to 12 months, while recent sex-chromatin studies by Chi and others (1965) revealed endothelial survival of up to 21 months. The survival of endothelial cells in the unfrozen grafts in the present study was 20, 20, and 32 months, which supports the previous findings and suggests that long-term experiments carried out for more than 2 years should be the rule in future investigations. If the figure of 344 days calculated by Binder and Binder (1957) for the life span of the rabbit endothelial cell is accepted, then survival of graft endothelium for 32 months implies that the transplanted endothelium has the property of self-perpetuation and this is of considerable fundamental interest from the point of view of cellular immunity.

The phenomenon of 'partial' repopulation has then two interpretations. The first is that a slow creeping replacement is secondary to the death of the donor cells at the periphery of the graft (Espiritu and others, 1961; Chi and others, 1965), this process being continuous throughout the life of the graft. The second is that the inflow of host cells takes place at the time of grafting and that thenceforth the self-perpetuating donor cells present a barrier to further host-cell infiltration. Since neither of these possibilities can be proved by the present techniques, which give a still-life picture of the particular stage in the individual graft, further speculation is unprofitable, but this point should provide a basis for further experimentation.

The combination of histological and cytological study of the grafted cornea was of value, and in retrospect it is apparent that more information could have been obtained if the whole of the cornea had been sectioned, because much of the histological interest is focused on the presence of granulation tissue at the host-graft junction and this area is not disturbed by the stripping procedure. Essentially the histological results obtained in the present experiment are similar to those of the classic descriptions of Tudor Thomas (1933), Castroviejo (1937), and Offret (1955); all three described opacities in grafts in which Descemet's membrane was duplicated and separated by fibrous wedges, clear grafts being characterized by minimal structural abnormality and the absence of inflammatory tissue. Although caution must be exercised in the interpretation of the results obtained in the present experiment, it seems reasonable to suggest that duplication of Descemet's membrane is an expression of endothelial repopulation and that this is related to graft opacity only when the two layers are separated by fibrous tissue. This small piece of evidence adds support to the findings of Polack and others (1964), and Chi and others (1965) who observed host endothelium in a few clinically clear grafts.

The encouraging clinical results obtained when frozen and preserved corneae are used for animal homografts (Mueller and Smith, 1963; Mueller, 1964) and human homografts (Mueller, Casey, and Trevor-Roper, 1964) suggest that the endothelium is not extensively damaged by freezing. Indeed it has been shown that many types of cells cooled slowly and frozen in the presence of demethyl sulphoxide can be recovered alive after thawing (Lovelock and Bishop, 1959; Ashwood-Smith, 1961; Dougherty, 1962), and Smith and others (1963) have shown that the endothelial layers of corneae frozen for a month in these conditions were able to produce viable clones on tissue culture. It would seem from the findings in the present study that the endothelium can survive *in vivo* after freezing and preservation. The numbers are too small for any conclusions to be drawn about the length of time in preservative, although there is a suggestion that increasing the duration of preservation diminishes the chance of endothelial survival. Failure of survival after a month in preservative is not surprising, since there is good evidence to suggest that many cell types gradually deteriorate even when preserved at temperatures as low as those obtained by the use of solid carbon dioxide (Rowson, 1956; Pegg, 1964). The adoption of liquid nitrogen refrigerators providing storage temperatures of -150°C. to -196°C. has brought about a significant improvement in the survival of other cell types and may be expected to do so in the case of mammalian corneal endothelium (Smith and Parkes, 1951; Phan The Tran, Bender, Cao Xuan Chuan, and Buu Hoi, 1963). If the finding of endothelial survival after freezing in the present study is confirmed,

there appears to be no biological reason why long-term frozen corneal homotransplants should fail.

Summary

The *in vivo* survival of the donor endothelium of eleven clear corneal homografts in the rabbit has been studied, using sex-chromatin as a marker to identify host and donor cells.

Three control grafts were preserved at 4°C. for 4 hours, and the endothelium was shown to have survived for 20, 20, and 32 months.

Eight grafts were preserved in demethyl sulphoxide (7.5 per cent.) and glycerol for between 1 and 100 days, and in four of these there was evidence of survival of donor endothelium for periods of up to 24 months *in vivo*.

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REFERENCES

- ASHWOOD-SMITH, M. J. (1961). *Nature (Lond.)*, **190**, 1204.
 BINDER, R. F., and BINDER, H. F. (1957). *A.M.A. Arch. Ophthalm.*, **57**, 11.
 CASTROVIEJO, R. (1937). *Trans. Amer. ophthalm. Soc.*, **35**, 355.
 CHI, H. H., TENG, C. C., and KATZIN, H. M. (1965). *Amer. J. Ophthalm.*, **59**, 186.
 DOUGHERTY, R. M. (1962). *Nature (Lond.)*, **193**, 550.
 ESPIRITU, R. B., KARA, G. B., and TABOWITZ, D. (1961). *Amer. J. Ophthalm.*, **52**, 91.
 HANNA, C., and IRWIN, E. S. (1962). *Arch. Ophthalm. (Chicago)*, **68**, 810.
 KLINGER, H. P., and LUDWIG, K. S. (1957). *Stain Technol.*, **32**, 235.
 LOVELOCK, J. E., and BISHOP, M. W. H. (1959). *Nature (Lond.)*, **183**, 1394.
 MUELLER, F. O. (1964). *Brit. J. Ophthalm.*, **48**, 377.
 ———, CASEY, T. A., and TREVOR-ROPER, P. D. (1964). *Brit. med. J.*, **2**, 473.
 ——— and SMITH, A. U. (1963). *Exp. Eye Res.*, **2**, 237.
 OFFRET, G. (1955). In "Corneal Grafts", ed. B. W. Rycroft, p. 36. Butterworth, London.
 PEGG, D. E. (1964). *J. appl. Physiol.*, **19**, 301.
 PHAN THE TRAN, BENDER, M. A., CAO XUAN CHUAN, and BUU HOI, N. P. (1963). *Rev. franç. Ét. clin. biol.*, **8**, 589.
 POLACK, F. M., and SMELSER, G. K. (1962). *Proc. Soc. exp. Biol. (N. Y.)*, **110**, 60.
 ———, ———, and ROSE, J. (1964). *Amer. J. Ophthalm.*, **57**, 67.
 ROWSON, L. E. A. (1956). "Proc. III Int. Congr. Animal Reproduction". June, 1956, Cambridge.
 SMITH, A. U., ASHWOOD-SMITH, M. J., and YOUNG, M. R. (1963). *Exp. Eye Res.*, **2**, 71.
 ——— and PARKES, A. S. (1951). *Lancet*, **2**, 570.
 STOCKER, F. W., EIRING, A., GEORGIADIS, R., and GEORGIADIS, N. (1958). *Amer. J. Ophthalm.*, **46**, no. 1, pt II, p. 294.
 THOMAS, J. W. TUDOR (1933). *Brit. J. Ophthalm.*, **17**, 529.