ORIGINAL ARTICLES—Laboratory science

Effect of ABO blood group mismatching on corneal epithelial cells: an in vitro study

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Abstract

***Aim*—To determine, in vitro, the effects of blood group ABO mismatching on corneal epithelial cells.**

***Methods*—Corneal epithelial cell cultures were established from 32 human cadaver donor eyes. Epithelial cells (100 µl of 4×10^2 cells per µl) were incubated for 4 hours with antibodies against blood group antigens A, B, and AB, with and without complement. Cell lysis was assayed by a chemiluminescent assay using Cytolite reagent. Live cells, remaining after incubation, were counted in a scintillation counter. The blood group of the donors was determined retrospectively, in a blinded manner.**

***Results*—Retrospective tracing of donor blood groups was possible for 20 donors. In all cases the blood group corresponded with that suggested by the cell lysis assay. Significant cell lysis was observed when known A group cells were incubated with anti-A and anti-AB antibody, B group cells were incubated with anti-B and AB antibody, and AB group cells were incubated with anti-AB antibody. Lysis occurred only in the presence of complement. No lysis of O group cells was observed with any of the antibodies. In all cases, lysis was observed only with neat (serum) antibody concentrations.**

***Conclusions*—Blood group ABO mismatching results in significant lysis of corneal epithelial cells. The antibody concentration required for lysis equals that found in serum. Such levels of antibody are unlikely to be achieved in tears and/or aqueous. This may offer an explanation for the conflicting reports of the studies on the effect of blood group matching on corneal grafts. The variability in the outcome may reflect the levels of antibodies gaining access to the corneal cells and not the mismatching alone.**

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Corneal transplantation is a very commonly performed solid organ transplantation procedure. It is estimated that over 40 000 corneal transplants are performed annually in the United States[1] and between 37 and 38 per million population in the United Kingdom.[2] Despite the relatively high success rate enjoyed by the corneal graft recipients, immunological graft rejection remains a significant cause of graft failure.[3 4] It is estimated that irreversible immunological rejection accounts for at least 33% of all graft failures.[5 6] Regrafts, for previously failed grafts, are rapidly becoming an important indication for corneal grafting. In one study, regrafting accounted for 18% of all graft procedures, placing it second only to pseudophakic bullous keratopathy (24%).[7]

The antigenic systems most studied in allograft rejection are the HLA system and the ABO blood group system. The expression of both HLA antigens and ABO blood group antigens in human cornea is well established.[8–11] However, the role of tissue and blood group matching to minimise corneal graft rejection is not clear. There have been several clinical studies that indicate that HLA class 1 matching confers a survival advantage in high risk cases,[12–15] but other studies do not support this observation. Clinical data on the relevance of HLA-DR and ABO blood group matching in graft survival are also ambiguous and studies have produced mixed results.[4 13 16–24]

The prospective double masked multicentre, study reported by the Collaborative Corneal Transplantation Studies Research Group (CCTS) in 1992 concluded that: (1) neither HLA-A, HLA-B nor HLA-DR antigen matching substantially reduced the likelihood of corneal graft failure; (2) a positive donor-recipient (HLA) crossmatch does not dramatically increase the risk of corneal graft failure; and (3) ABO blood group matching may be effective in reducing the risk of graft failure. Following the report of the CCTS, other reports on the effects of ABO matching on corneal graft survival were published. While some found no correlation between ABO matching and graft survival,[20] one group of investigators found a higher rate of graft survival with ABO matching in high risk patients with vascularised corneas or a previous episode of irreversible graft rejection.[21]

In 1998 we provided preliminary evidence to demonstrate in vitro agglutination of corneal epithelial cells following ABO mismatching.[25] The aim of the present study was to ascertain whether ABO mismatching causes corneal epithelial cell lysis in vitro and to quantify the degree of cell lysis in relation to the concentration of antibody applied.

Methods

PRIMARY EXPLANT CULTURE

Corneal epithelial cell cultures were established from human donor corneoscleral rims following the method described by Jumblatt *et al*.[26] Primary explant cultures were grown in six well Primaria tissue culture plates (Becton Dickinson, Oxford, UK) with growth media (Hanks F12 and HEPES buffered Dulbecco's modified Eagle's medium 1:1 (Gibco BRL, Life Technologies, Paisley, UK); supplemented with 5% fetal bovine serum (Gibco); 0.5% DMSO (Sigma-Aldrich, Dorset, UK); 1 μg/ml gentamicin (Gibco); 10 ng/ml epidermal growth factor (Gibco); 5 μg/ml bovine insulin (Gibco); 5.65 μg/ml amphotericin B (Sigma), and 0.1 μg/ml cholera toxin (Gibco)). Cells were fed twice a week with fresh growth medium and on reaching confluence, they were harvested with Versene 0.05% solution (commercially prepared combination of trypsin 0.05% and EDTA 0.05%, Sigma, UK). The cells were then resuspended in growth medium to give a single cell suspension and the number of live cells (trypan blue dye exclusion) were counted with a haemocytometer. A mouse monoclonal anti-cytokeratin-3 antibody, AE5 (ICN Biomedicals, Inc, Costa Messa, CA, USA) was used to confirm the epithelial nature of cells used in the study.

CYTOTOXICITY ASSAY

Corneal epithelial cells were incubated with donor antiserum with and without complement. The antiserum used was obtained from multiple donors of known blood groups. Pooled blood group antisera were obtained from the National Blood Transfusion Service, Sheffield. The serum obtained was tested against reagent red blood cells to establish the presence of blood group antibodies. The serum complement was not heat inactivated. To obtain antiserum with antibodies devoid of complement a portion of the serum was heat inactivated at 56°C for 30 minutes.

A volume of 100 μl of cells (containing 4×10^2 cells per μl) was incubated in 96 well microtitre plates in quadruplicates sets containing each of the following: (1)100 μl of saline, (2) 100 μl of antiserum against A, (3) 100 μl of antiserum against B, (4) 100 μl of antiserum against AB. The antiserum in sets 2–4 was heat inactivated, (5) 100 μl of antiserum against A (with complement), (6) 100 μl of antiserum against B (with complement), (7) 100 μl of antiserum against AB (with complement). The antiserum in sets 5–7 was not heat inactivated. These cells were incubated at 37°C in 5% carbon dioxide and 95% air for 4 hours and then assessed for cell lysis.

ASSESSMENT OF CELL LYSIS

Various methods were evaluated as these corneal epithelial cells are less robust than most types of epithelial and haematopoietic cells. Hence methods which can be employed on most cell types were found to be unsuitable for corneal cells. The intended chromium release assay proved unsuitable after various attempts, as the cells were unable to survive the labelling process and the multiple washes that were required by this method. Lactate dehydrogenase (LDH) release was not sensitive enough owing to the fact that in our system, antiserum, which was the incubating medium, contained too high a level of LDH itself thus producing a background level, which obscured any peaks of LDH that might have been released by the cells into the system; 100% killing was required for any sort of reproducible and reliable detection, hence lesser degrees of lysis were undetectable. This severely compromised the sensitivity of this method for our purpose. Detection of cell damage was attempted by staining with propidium iodide and passing cells through the flow cytometer but establishing the proper parameters for gating was difficult owing to the heterogeneity of the size of these cells. Furthermore the numbers needed for flow cytometry, as dictated by our design, were far too large to be generated by a single corneal rim.

Cell lysis was detected by a chemiluminescent assay using Cytolite reagent (Canberra Packard, Berks, UK). The assay detects live cells, by causing them to fluoresce but not dead cells. Cytolite is a commercially available reagent produced by Canberra Packard as a chemiluminometric alternative to colorimetric, fluorometric, and radioisotopic assays to quantify viable cells. It has been used for the quantitative evaluation of proliferation of, and cytotoxicity effects on, eukaryotic cells. The kit was used as per the manufacturer's instructions. Briefly, the kit is composed of two solutions—an "activator" solution containing a chemiluminogenic probe (CLP), and an "amplifier" solution containing a reduced coenzyme in a proprietary "carrier" solution. In suspension, eukaryotic cells, with an intact cell membrane, possess a net negative surface charge. As a result, cationic CLP will bind to the cell surface. Upon contact, the CLP^{2+} is transformed into a univalent radical state, CLP^{+}. The CLP is present in the activator solution in excess, thereby allowing all intact cell membranes to become fully saturated with the probe. This activation process of CLP binding and transformation to the reduced CLP^{+} state is stable and rapid in room temperature. Free unbound CLP remains in the CLP^{2+} state and does not participate in the reaction process further.

The reduced coenzyme, contained in the amplifier solution, is taken up by the cells. The reduced coenzyme drives electron transferring reactions wherein oxygen is the final acceptor. The result of this pathway is the generation of reactive oxygen species (ROS) which can diffuse freely out of the cell. The ROS reacts with the CLP^{+} on the cell membrane and an intermediate is formed which spontaneously decomposes producing long lived glow luminescence. The light produced is proportional to the number of viable cells. The cells were counted in a Top-Count scintillation counter (Canberra Packard, Berks, UK) which detected the fluorescence.

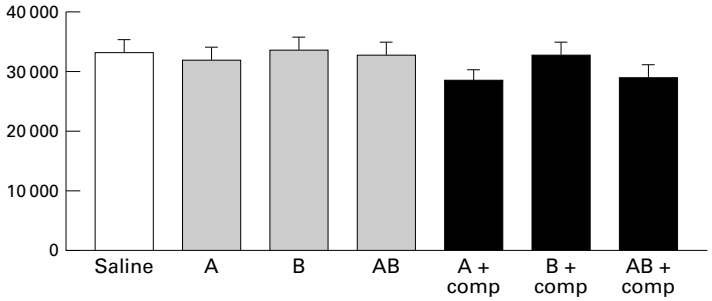

Figure 1 Shows live cells remaining after blood group A corneal epithelial cells were incubated with the various antisera, with and without complement. Significant lysis was observed with anti-A + complement and anti-AB + complement. (A = anti-A antibody, B = anti-B antibody, AB = anti-AB antibody, comp = complement.) The Y axis represents scintillation counts (0.02 minutes/well) of live cells. Error bars represent SEM.

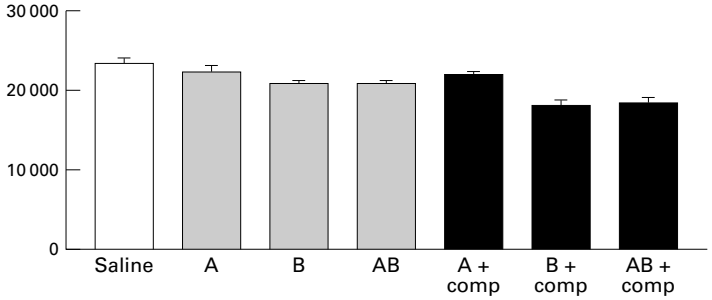

Figure 2 Shows live cells remaining after blood group B corneal epithelial cells were incubated with the various antisera, with and without complement. Significant lysis was observed with anti-B + complement and anti-AB + complement. (A = anti-A antibody, B = anti-B antibody, AB = anti-AB antibody, comp = complement.) The Y axis represents scintillation counts (0.02 minutes/well) of live cells. Error bars represent SEM.

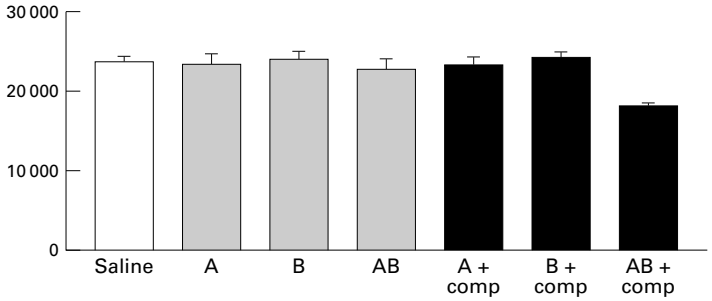

Figure 3 Shows live cells remaining after blood group AB corneal epithelial cells were incubated with the various antisera, with and without complement. Significant lysis was observed with anti-AB + complement only. (A = anti-A antibody, B = anti-B antibody, AB = anti-AB antibody, comp = complement.) The Y axis represents scintillation counts (0.02 minutes/well) of live cells. Error bars represent SEM.

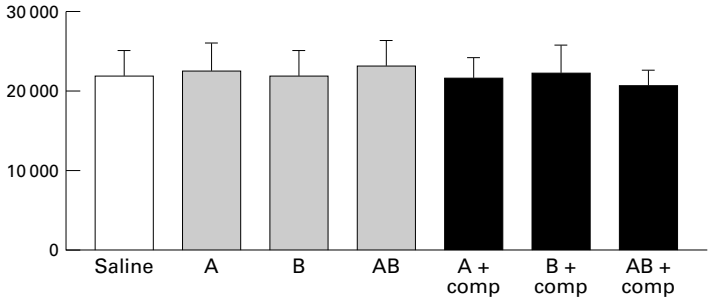

Figure 4 Shows live cells remaining after blood group O corneal epithelial cells were incubated with the various antisera, with and without complement. Lysis was not detected. (A = anti-A antibody, B = anti-B antibody, AB = anti-AB antibody, comp = complement.) The Y axis represents scintillation counts (0.02 minutes/well) of live cells. Error bars represent SEM.

CALIBRATION AND STANDARD CURVE

After harvesting the cultured corneal epithelial cells to obtain a single cell suspension, serial dilutions (eight 1:2 serial dilutions) in quadruplicates of 100 µl of Cytolite reagent/well were set up in 96 well microlitre plates to obtain a standard curve. As per manufacturer's instructions, three sets of these quadruplicate serial dilutions were set up for the calibration procedure to determine the most appropriate volume of amplifier solution to be used for the standard curve. The amplifier solution that produced the best standard curve was also used for the assessment of cell lysis in the subsequent cytotoxicity assay. In this case 100 µl of amplifier solution was found to be the most appropriate.

CELL LYSIS ASSESSMENT WITH CYTOLITE

Cells cultured from 32 donor corneoscleral rims were assessed for lysis. After 4 hours of incubation with different antisera as described above, 25 µl of activator solution and then 100 µl of amplifier solution were added to each well. The microplates were then passed through the Top-Count scintillation counter for 0.02 minutes/well at 25°C to obtain the luminescent counts of each well. The total cell counts were obtained by reading off from the standard curve.

RETROSPECTIVE TRACING OF THE BLOOD GROUP IDENTITY OF THE CORNEAL DONORS

The blood group of the donors was traced retrospectively after the experiments had been completed. The UKTSSA (United Kingdom Transplant Service Support Authority) has records of the blood groups of all multiorgan donors. Blood groups of other donors were traced from the blood banks of the donor hospital or from the records of general practitioners with whom the donor was registered when alive. The study was conducted in a "blind" manner with the observer having no previous knowledge of the donor's blood group. The tenets of the declaration of Helsinki were adhered to and institutional ethics committee approval was obtained.

CONTROLS

Beside saline, the anti-A antiserum for (eye) donors of blood group B, the anti-B antiserum for (eyes) donors of blood group A, the anti-A and anti-B antiserum for (eye) donors of blood group O and for (eye) donors of blood group AB, also served as controls.

Results

The scintillation counts of corneal epithelial cells with Cytolite conformed to a normal pattern of distribution and the standard curve obtained was reproducible. The standard curve showed a linear correlation between scintillation counts and cell counts.

The 32 samples of cultured corneoscleral rims were grouped, according to where cell lysis occurred, into four groups: cell lysis occurred in 10 samples of cells incubated with anti-A (Fig 1), seven samples with anti-B (Fig 2), and seven samples with anti-AB (Fig 3). Eight samples showed no lysis with any of the antisera tested (Fig 4).

In the first group that showed lysis with anti-A antiserum plus complement, all 10 cases also demonstrated lysis in blood group

AB antiserum with complement but not with anti-B antiserum with complement. No lysis was demonstrated with any antiserum without complement or with saline. In the second group of seven samples that showed lysis with anti-B antiserum plus complement, all samples also demonstrated lysis with anti-AB antiserum plus complement but not in A antiserum with complement. Again no lysis was detected in any of the antisera without complement or with saline. In the third group of seven cases that showed lysis with anti-AB antiserum with complement, no lysis was found with any other antiserum either with or without complement.

The blood groups of 20 donors were traced retrospectively. The blood groups of the remaining 12 donors could not be traced despite all efforts. In the first group, 10 donor rim samples showed lysis with anti-A and anti-AB. Eight of these could be traced and all eight were of blood group A. In the second group, seven samples demonstrated lysis with anti-B and anti-AB. Only four could be traced. Two were of blood group B and two of blood group AB. In the third group, seven samples showed lysis with anti-AB only. Four of these could be traced and all four were found to be of blood group AB. In the fourth group, eight samples had demonstrated no lysis with any antiserum. Six could be traced and all six were of blood group O. For the four groups, the results obtained were analysed by multistep analysis of variance (ANOVA).

Only the samples, which were successfully traced, were included in the analysis. Where a significant difference in cell counts were indicated by the multistep ANOVA ($p<0.05$), a Tukey's test was applied to compare for significant differences ($p<0.05$) between all possible combinations within each group. In these first three groups, significance was demonstrated with multistep ANOVA ($p<0.05$) and Tukey's test ($p<0.05$). In the fourth group, which showed no lysis, there was no significance shown with ANOVA for the group and therefore it excluded the application of Tukey's test. The data were analysed with a statistics program called PRIZM 2. The average degree of lysis observed was 15%. The maximum degree of cell lysis detected was approximately 30%. Lysis was detected with neat serum only. At lesser serum concentrations (1:2 and 1:4) no evidence of lysis was detected by this assay.

Discussion

The avascularity of the cornea and lack of lymphatic drainage channels have traditionally been considered to confer a state of "immune privilege" on the cornea. Other factors such as a paucity of antigens presenting (Langerhans) cells from the central cornea, intracorneal production of immunosuppressive factors (TGF-β and others).[27] The phenomenon of anterior chamber associated immune deviation (ACAID)[28] and conjunctiva associated lymphoid tissue (CALT) induced tolerance,[29 30] all make immune privilege in the eye, an active and dynamic process. However, it is now well established that this privilege is not absolute and immune mediated graft rejection is still an important cause of graft failure.[3]

Both MHC and non-MHC antigens have been implicated in corneal graft rejection.[24 31 32]

Recent attention has (re)focused on the blood group antigens as important among the non-MHC antigens. In general, transplanting across ABO blood groups has been associated with a higher degree of rejection in all vascularised organs, such as kidney, heart, and liver. It has however also been observed that the risk of transplanting across blood groups is not absolute. For example, the transplantation of blood group A2 kidneys into blood group O recipients has not been reported to produce a significantly increased risk of graft loss.[33] It is also well known that a difference in susceptibility to ABO mismatches exists between different organs. Liver and bone marrow transplants have been noted to be less susceptible to ABO incompatibility between donor and recipient than kidney and cardiac transplants and therefore transplantation of the bone marrow and liver in particular are sometimes performed across blood groups, in urgent cases where there is a shortage of compatible blood group donors.[34 35]

The few studies on the influence of ABO blood group matching on corneal graft rejection have produced rather mixed results. Allansmith *et al*[17] reported an overall failure rate of 8% for ABO matched corneal grafts and 11% for mismatched corneal grafts out of a total of 150 grafts. Batchelor *et al*[22] reported that ABO blood group matching had no effect on graft survival. It was suggested by these investigators in the earlier part of the 1970s that a larger series with a higher failure rate might be needed to show the effects of ABO blood group incompatibility on graft survival.[22] In a much larger study, Meyer *et al*[36] included 250 cases with a higher overall failure rate of 25%, and found no relation between ABO incompatibility and graft failure. A study with an even higher failure rate of 68%, by Mehri *et al*,[19] also found no effect of ABO matching on graft failure. They however had only a total of 68 cases. Further, Boisjoly *et al*[13] found no effect of ABO compatibility in their series of 250 cases. A rather interesting study in 1982 by Volker-Dieben *et al*[14] reported that in comparison, type A corneas transplanted into type O recipients showed a better 1 year survival rate than type A corneas transplanted into type A recipients. However, there was no significant difference in graft survival at 1 year between type A recipients of type O corneas and type O recipients of type O corneas. It has been difficult to compare the findings of these studies for a number of reasons. Firstly, different primary outcome measures, such as frequency of rejection episodes, graft survival, 1 year graft survival rates, and 1 year rejection free survival rates, were considered. More importantly, there was great variation in the dosages of topical steroid used postoperatively. Furthermore, the most common criticism of some of these studies was that they were not double blind prospective randomised trials. Although some of these studies had attempted to look into and

control for various risk factors, none of these studies controlled for the presence of HLA mismatching.

The Collaborative Corneal Transplant Study attempted to address these limitations of previous studies. A total of 419 patients were studied in a double blind manner. The study was designed to investigate the effects of HLA-A, HLA-B, and HLA-DR matching on corneal graft survival. ABO compatibility was determined but not used for recipient selection. All recipients were grouped into high risk and low risk groups based on the number of HLA matches. Graft rejection in ABO compatible and incompatible groups were compared within these groups controlling for the potential effects of HLA matching on the final outcome measures. The study concluded that HLA was found to have no effect on graft survival. However, overall graft failure rates of 31% and 41% were reported for the ABO compatible and ABO incompatible groups respectively and graft failure rate from rejection was estimated at 16% and 30% for ABO compatible and ABO incompatible groups, respectively. They suggested that while HLA matching had shown to be non-beneficial, ABO matching might perhaps be of benefit.[4]

Borderie *et al*[21] in 1997 evaluated the effects of ABO compatibility on high risk transplants defined as recipients with vascularised corneas or recipients with previously rejected grafts in comparison with low risk groups. They reported that 1 year rejection-free survival rates were significantly higher in the ABO compatible group than in the incompatible group and concluded that ABO matching may be effective in preventing irreversible rejection in these high risk groups. However, other single centre trials in the same year reported no correlation between the effects of ABO matching and graft failure.[20]

In a preliminary study we had demonstrated that cultured corneal epithelial cells readily agglutinated when treated with antibody against their respective blood group.[25] In the present study, we observed lysis when corneal epithelial cells of blood group A and B donors were incubated in antiserum against A and B antigen respectively, only when the antiserum was not heat inactivated—that is, contained complement. Lysis was also observed with these cells in AB antiserum (not heat inactivated). Blood group identity on retrospective tracing revealed the (eye) donors were indeed from blood group A and blood group B respectively. The observation that blood group AB antiserum is able to cause lysis of corneal epithelial cells of donors of blood group A and B is probably due to the fact that AB antibodies do have affinity for binding to both A and B antigenic sites and are hence able to produce damage by initiating the complement cascade.

Interestingly, however, the converse was not true. In the presence of complement, blood group AB corneal epithelial cells demonstrated significant lysis only when incubated in antiserum against blood group AB but not with antiserum against A alone or B alone. This may perhaps be due to some differences in antigen density on the cells and therefore to the amount of antibody binding and consequent complement activation.

However, the degree of lysis that did occur was observed to be quite small with a maximum amount of only 30%. Furthermore, this occurred only with neat serum indicating that high concentrations of antibody are required to adversely affect corneal epithelial cells. Halving the neat serum concentration levels did not produce any detectable lysis in our study. This observation may be of relevance to the clinical observation that corneal grafts are successful even without blood group matching when the blood-ocular barrier remains undisrupted. The level of immunoglobulins present in the tears[37] and aqueous[38] is several times lower than in serum. Furthermore, immunoglobulins of the IgA, IgG, and IgE class are present in tears to any significant extent. Similarly only IgG, and in some cases IgA, are significant immunoglobulins constituents of the aqueous. Blood group antibodies are mainly of the IgM class, which is generally not detectable in tears and aqueous.[37 38] Thus, even though epithelial cell agglutination with associated cell death[25] and cell lysis as shown in this study can occur by ABO mismatching, it is unlikely that these can occur in vivo. These observations with regard ABO mismatching and corneal epithelium, add another dimension to the "immune privilege" of the cornea. Blood group antigens have been demonstrated on the corneal endothelium.[10 11] It is therefore tempting, though not directly possible, to extrapolate these findings to the endothelium as well.

It is well known that when the corneal bed becomes vascularised or when there is active inflammation, corneal grafts are at high risk of rejection. In such situations it is possible for immunoglobulins to gain access to the cornea via the tears, through the invading vessels or following the break down of the blood-aqueous barrier. In these cases, antibodies against blood group ABO antigens could contribute to cell damage.

This may offer an explanation for the conflicting reports of studies on the effects of blood group matching on corneal grafts. The variability in the outcome may reflect the levels of antibodies gaining access to corneal cells and not the mismatching alone.

This study has demonstrated that blood group ABO mismatching does cause corneal epithelial cell lysis in vitro. However, a high concentration of antibody and presence of complement was required to produce a small amount lysis. These conditions are unlikely to be met in clinical situations, especially in non-vascularised corneas and when the blood-ocular barrier is intact (or only temporarily disrupted as in the immediate post-graft period). This could be one explanation for the clinical observation that most corneal grafts survive despite mismatches across the ABO blood groups.

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