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BENEFICIAL EFFECT OF MATCHING AT THE HLA-A and -B AMINO-ACID TRIPLET LEVEL ON REJECTION-FREE CLEAR GRAFT SURVIVAL IN PENETRATING KERATOPLASY[1]

DANIEL BÖHRINGER,[2,8] THOMAS REINHARD,[2] RENÉ J. DUQUESNOY,[3] STEFAN BÖHRINGER,[4] JÜRGEN ENCZMANN,[5] PETER LANGE,[6] FRANS CLAAS,[6] AND RAINER SUNDMACHER[7]

Objective. **The beneficial effect of human leukocyte antigen (HLA) matching on long-term prognosis in penetrating keratoplasty is now unequivocal but has to be weighed against the additional waiting period on an individual basis. HLAMatchmaker is a molecularly based algorithm for histocompatibility determination that can identify immunologically acceptable mismatches and thus potentially reduce time on the waiting list dramatically without negatively affecting prognosis.**

Methods. **The HLAMatchmaker algorithm (triplet-string matching) was applied on each of 545 normal-risk keratoplasties for which complete HLA type was known at split-level resolution. Two homogenous groups were defined. Group I consisted of the 147 penetrating keratoplasties with up to 13 triplet-string mismatches (the typical upper limit of foreign in case of a single HLA-A or HLA-B allele mismatch) and was compared to the remaining 398 patients with more triplet mismatches (group II) using the Kaplan-Meier method and log-rank statistics. Analysis of clear graft survival on the basis of conventional HLA-A and HLA-B matching was performed as well. Reduction of time on the waiting list as compared to conventional HLA-A and HLA-B matching was predicted individually.**

Results. **Triplet-string matching yielded 85% rejection-free clear graft survival 3 years after penetrating keratoplasty in group I but only 76% in group II ($P<0.05$), whereas conventional HLA-A and HLA-B matching did not result in any statistically significant reduction of immune reactions because of lack of statistical power ($P=0.08$). Triplet-string matching (13 mismatches accepted) reduces median time on the waiting list by 80%.**

Conclusions. **Triplet-string matching seems to improve mid- to long-term prognosis in penetrating keratoplasties while simultaneously reducing time on the waiting list in most cases. It should thus be considered for histocompatibility determination in penetrating keratoplasty.**

The beneficial effect of human leukocyte antigen (HLA) matching on long-term prognosis in penetrating keratoplasty is now unequivocal (*1–3*). Nevertheless, it is still neglected in most eye clinics, because any improvement of prognosis has to be weighed against time on the waiting list resulting from the search for a histocompatible donor. The delay caused by this search is associated with reduced quality of life (*4*) and, if disease is symmetric, social costs of blindness as well (*5*). Variability of this delay is high but can be predicted on an individual basis (*6*). Waiting periods strongly depend on the count of accepted HLA mismatches: very long waiting periods are mostly observed when seeking complete HLA matches. When at least one mismatch is accepted, time on the waiting list is shorter than 2 years for almost all individuals (*6*).

The fact that certain HLA mismatches differ in strength of immunogenicity and thus in deterioration of graft survival has been known for sometime in kidney transplantation (*7*) and in keratoplasty (*8*). For HLA class I loci, the structural basis of this phenomenon has recently been established (*9–12*). This paves the way for predicting acceptable mismatches on an individual basis: the HLAMatchmaker algorithm applies the concept that each HLA antigen consists of multiple exposed (accessible to antibodies and receptors of killer cells) epitopes that can elicit specific immune responses such as alloantibodies. These polymorphic epitopes are well defined by their position within the HLA molecule and partitioned into triplets of amino acids. A few triplets show an overlap of a monomorphic amino acid residue but are counted as individual triplets because antibodies to such triplet-associated epitopes have been identified (*11*). The degree of matching is assessed by determining whether or not each triplet of the mismatched HLA antigen is also found in the corresponding positions within any of the patient's own HLA-A and HLA-B molecules. The algorithm can thus identify mismatched HLA antigens without any mismatches at the triplet level for each donor-recipient combination. Although formally HLA mismatches, these donor HLA alleles are supposed to be fully histocompatible with regard to the antibody epitopes, and are shown not to cause deterioration of graft survival in kidney transplantation (*10*). This finding is remarkable because most epitopes recognized by T cells or antibodies are potentially more complex than foreign amino-acid triplets on HLA mismatches. In penetrating keratoplasty, where immunologic mechanisms are potentially different from other solid organ transplantations (*13*), no structural approach toward

[1] Supported by Bio Implant Services Foundation, Leiden, The Netherlands.

[2] Eye Hospital and LIONS Cornea Bank Baden-Württemberg, University Hospital, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany.

[3] University of Pittsburgh Medical Centre, Pittsburgh, PA.

[4] Institute for Human Genetics, University Essen, Essen, Germany.

[5] Institute for Transplantation Immunology, University Hospital, Heinrich-Heine-University, Düsseldorf, Germany.

[6] University Medical Centre, Leiden, The Netherlands.

[7] Eye Hospital and LIONS Cornea Bank North Rhine Westphalia, University Hospital Heinrich-Heine-University, Düsseldorf, Germany.

[8] Address correspondence to: Daniel Böhringer, M.D., Eye Hospital, University Hospital, Freiburg, Killianstr. 5, D-79106 Freiburg, Germany. E-mail boehringer@aug.ukl.uni-freiburg.de.

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differential immunogenicity of different HLA mismatches has yet been formulated. The purpose of this study was to test whether application of the HLAMatchmaker algorithm is of benefit in penetrating keratoplasty with respect to both graft prognosis and associated reduction of time on the waiting list.

PATIENTS AND METHODS

Patient Selection

Penetrating normal-risk keratoplasty was performed in a homogenous single-center group of 545 patients after obtaining written informed consent from every patient. Only eyes with avascular host cornea undergoing first keratoplasty were considered. Indications for surgery were keratoconus, Fuchs' endothelial dystrophy, bullous keratopathy, and nonherpetic avascular scars. None of the patients had a history of severe surface disorders, glaucoma, or herpetic eye disease.

HLA Typing and Matching Strategy

HLA typing of all donors and recipients was performed serologically for HLA-A and HLA-B and at the molecular level for HLA-DR in a single laboratory accredited by the American Society for Histocompatibility and Immunogenetics (*14*).

All patients were placed on the waiting list for an HLA-compatible graft by means of conventional HLA matching (A, B, and DR) at broad-level resolution for a maximum of 6 to 8 months. If a graft exerting up to one mismatch at the loci HLA-A, HLA-B, and HLA-DR (broad resolution) was not found within that time interval, the next available graft was assigned, regardless of number of HLA mismatches.

Triplet-string matching was retrospectively performed by custom software implemented in the computer language Perl 5.6. A Microsoft Access 2000 (Microsoft Corp., Redmond, WA)-based triplet string matching program was also developed for validating the Perl program. Software is available through the authors free of charge on request.

Triplet strings were predicted from individual HLA types at split level by assigning the triplets corresponding to the most common, molecularly defined HLA allele for the corresponding HLA split. For HLA-B44 and HLA-B35, where more than one allele occurs relatively commonly, triplets of HLA-B*3501 were arbitrarily assigned to HLA-B35 and that of HLA-B*4402 to HLA-B44.

Stratification and Statistical Analysis

The relative risk for developing a graft rejection resulting from each mismatch at the triplet-string level was determined by means of Cox proportional hazards regression analysis. In addition, three Kaplan-Meier estimations of rejection-free clear graft survival after 1,200 days (3.3 years) were calculated to estimate a reasonable match grade at the triplet level in penetrating keratoplasty. Grouping criteria were maximum (n=13) and mean count (n=6) of triplet-string mismatches as observed in a single HLA mismatch in this study. Last but not least, conventional HLA-A and HLA-B matching was performed.

Patient, organ culture, and donor data are summarized in Tables 1 through 3. Groups in all three approaches are mostly comparable with respect to established influencing factors on clear graft survival. An important exception is match grade at HLA-DR. Furthermore, grafts from the poorly matched groups tend to be kept in organ culture slightly shorter. This small variation in storage time in organ culture is not known to be an influencing factor on rejection-free clear graft survival, however (*15*, *16*).

The log-rank test was applied for testing the Kaplan-Meier estimations for statistical significance. All statistical evaluation was performed using SPSS 11 (SPSS, Inc., Chicago, IL) on Windows XP (Microsoft).

Prediction of Time on the Waiting List

A mathematical model derived from survival analysis was used to predict time on the waiting list (*6*): waiting time is reciprocally related to the total percentage of suitable donors in the donor population, which in turn depends on the accepted count of HLA mismatches and on the individual HLA phenotype. It is calculable from a database consisting of the most common HLA haplotype frequencies in the donor population (Bone Marrow Donors Worldwide data for Germany with frequencies exceeding 0.01% of the population). The total percentage of suitable donors is calculated by summing all phenotype frequencies (numeric products of all permutations of the appropriate haplotype frequencies) that meet the matching criterion such as up to six or up to 13 triplet-string mismatches (Table 4). The much simpler approach of calculating potential waiting time from allele frequencies instead of haplotype frequencies would yield erroneous results because of strong linkage disequilibrium between the HLA loci: only a small fraction of all possible haplotypes actually exists in a specific population. This approach would thus underestimate expected time on the waiting list. Reciprocal total percentage of suitable donors is adjusted for the daily rate of new donors and other local parameters (*6*, *17*), yielding expected waiting time.

TABLE 1. Triplet-string matching: patient, organ culture, and donor data[a]. Six mismatches at the triplet-string level.

	Group I	Group II	*P* value
No.	51	494	—
Percentage with up to one HLA-DR mismatch	78.4	46.6	<0.01[c]
KK/FD/BKP/other (%)	35.3/31.4/27.5/5.8	28.9/36.4/21.9/12.8	0.36[c]
Age of patients (yr)	56.2±20.6	59.5±19.4	0.10[b]
Female/male patients	30/21	270/224	0.34[c]
Age of donors (yr)	62.8±16.1	62.9±17.1	0.94[b]
Female/male donors	26/25	197/297	0.08[c]
Postmortem time (hr)	13.1±13.9	14.84±16.0	0.95[b]
Storage time in organ culture (days)	17.6±4.3	15.0±16.2	0.31[b]
Endothelial cell density after organ culture (cells/mm^2)	2,324.05±228.5	2,329.1±245.5	0.9[b]
Follow-up (days)	893.5±616.8	709.9±544.9	0.02[b]

[a] Group I consists of those keratoplasties with only up to six mismatches at the triplet-string level, and group II consists of the remaining less well-matched keratoplasties.

[b] *t* test.

[c] χ^2 test, mean±SD.

FD, Fuchs' dystrophy; KK, keratoconus; BKP, bullous keratopathy; other, nonherpetic corneal scars.

TABLE 2. Triplet-string matching: patient, organ culture, and donor data[a]. Thirteen mismatches at the triplet-string level.

	Group I	Group II	*P* value
No.	147	398	—
Percentage with up to one HLA-DR mismatch	59.9	45.7	0.02[c]
KK/FD/BKP/other (%)	34.0/33.3/21.8/10.9	27.9/36.9/22.6/12.6	0.22[c]
Age of patients (yr)	56.4±21.0	59.5±19.4	0.10[b]
Female/male patients	80/67	220/178	0.92[c]
Age of donors (yr)	62.5±18.1	62.6±16.6	0.96[b]
Female/male donors	62/85	161/237	0.39[a]
Postmortem time (hr)	14.7±15.9	14.84±16.0	0.95[b]
Storage time in organ culture (days)	17.6±4.3	17.2±4.4	0.32[b]
Endothelial cell density after organ culture (cells/mm^2)	2,340.5±290.4	2,324.1±223.8	0.55[b]
Follow-up (days)	795.7±582.6	727.1±554.0	0.079[b]

[a] Group I consists of those keratoplasties with only up to thirteen mismatches at the triplet-string level, and group II consists of the remaining less well-matched keratoplasties.
[b] *t* test.
[c] χ^2 test, mean±SD.
FD, Fuchs' dystrophy; KK, keratoconus; BKP, bullous keratopathy; other, nonherpetic corneal scars.

TABLE 3. Conventional HLA-class I matching (one mismatch at HLA-A/B loci): patient, organ culture, and donor data[a]

	Group I	Group II	*P* value
No.	57	488	—
Percentage with up to one HLA-DR mismatch	71.9	46.9	<0.01[c]
KK/FD/BKP/other (%)	36.8/35.1/22.8/5.3	28.7/36.1/22.3/12.9	0.67[c]
Age of patients (yr)	55.1±20.4	59.1±19.7	0.16[b]
Female/male patients	35/22	265/223	0.33[c]
Age of donors (yr)	64.0±15.5	62.4±17.2	0.50[b]
Female/male donors	30/27	193/295	0.06[c]
Postmortem time (hr)	13.2±13.0	15.0±16.3	0.43[b]
Storage time in organ culture (days)	18.7±3.7	17.13±4.5	0.01[b]
Endothelial cell density after organ culture (cells/mm^2)	2,308.4±219.3	2,331.3±246.8	0.54[b]
Follow-up (days)	811.7±553.8	717.2±553.8	0.22[b]

[a] Group I consists of those keratoplasties with only up to one mismatch at HLA-A/B loci, and group II consists of the remaining less well-matched keratoplasties.
[b] *t* test.
[c] χ^2 test, mean±SD.
FD, Fuchs' dystrophy; KK, keratoconus; BKP, bullous keratopathy; other, nonherpetic corneal scars.

TABLE 4. Waiting time and improvement of rejection-free clear graft survival

Grouping criterion	More than one conventional HLA mismatch A/B	More than 13 triplet mismatches	More than 6 triplet mismatches	More than 6 triplet mismatches[a]
Median time on the waiting list relative to conventional matching (%)	100	20	28	Not calculated
Improvement of clear graft survival after 1,200 days (%)	16 (NS)	9 ($P<0.05$)	15 (NS)	12 (NS)

[a] Only keratoplasties with at least one mismatch on HLA-DR.
NS, Not statistically significant.

Prediction of waiting time for a conventional HLA match (*6*) (HLA-A and HLA-B loci) was performed as well for every patient in this study accordingly. Both waiting periods were compared using the paired Wilcoxon test. Custom software was implemented in the computer language Perl 5.6 and is freely available on request.

Grafts

Only donor corneas that met the European Eye Bank Association criteria (*17*) were used for surgery. All corneas had been held in organ culture for at least 10 days. A postmortem time longer than 72 hr was an exclusion criterion. Data of donors are given in Tables 1 through 3.

Surgery

Penetrating keratoplasty was performed by only three experienced surgeons according to standardized procedures: a modified Franceschetti's trephine was used for trephination. The graft was cut from the endothelial (7.7 mm) side and the recipient cornea centrally from the epithelial (7.5 mm) side. The graft was temporarily fixed using nylon 10-0 sutures at the 3-, 6-, 9-, and 12-o'clock positions. Final fixation of the graft was performed with a double running diagonal suture (*18*) with 2×8 cross-stitches. The first suture was not removed before month 4 and the second one not before month 12 after keratoplasty. Suture removal was performed using topical anesthesia with proxymetacaine eye drops.

Medical Treatment

Directly after keratoplasty, both 0.5 mL of gentamicin 4% and 0.5 mL of dexamethasone 21-acetate 0.8% were injected subconjunctivally. Acetazolamide was administered orally to prevent a postoperative increase in intraocular pressure. One milligram of fluocor-

tolone per kilogram of body weight was routinely administered orally and tapered off in 2 weeks. Topical gentamicin 0.5% was applied at least five times daily until complete reepithelialization. Prednisolone 21-acetate 1% eye drops were administered five times daily during the first month, four times daily during the second month, three times during the third month, twice in the fourth month, and once in the fifth month postoperatively. No topical steroids were applied afterward.

Assessment of Rejection-Free Clear Graft Survival

Visits were scheduled at 6 weeks, 4 months, and 12 months postoperatively. Long-term follow-up was scheduled yearly.

Immune reactions were diagnosed by the presence of endothelial precipitates and stromal edema. All patients were treated with corticosteroid eye drops (prednisolone 21-acetate 1%) every hour, tapered individually until elimination of all precipitates was performed. Furthermore, a subconjunctival injection with betamethasone 21-acetate was performed. In severe cases, systemic corticosteroids at a daily oral dose of 1 mg of fluocortolone per kilogram of body mass were administered additionally and tapered within 3 weeks.

RESULTS

Cox proportional hazards analysis demonstrated a relative risk of 1.8 for developing a graft rejection (reversible or irreversible) per triplet-string mismatch ($P=0.05$). In Table 4, improvement of clear graft survival and waiting times are summarized for all three grouping approaches.

Statistical significance was observed only when comparing the group with up to 13 triplet-string mismatches to the poorly matched remaining keratoplasties (Fig. 1). Conventional HLA-A and HLA-B matching narrowly missed statistical significance in this study (Fig. 2).

For a graft with up to one conventional HLA match, the median predicted waiting period was 102.6 days (range, 8.54 days–49.7 years). For a graft with up to six triplet-string mismatches, it was 38.6 days (range, 6 days–15.6 years). When accepting 13 triplet-string mismatches, only 6.3 days (range, 1.78–166.69 days) of waiting time remained. These reductions were statistically highly significant according to paired Wilcoxon testing.

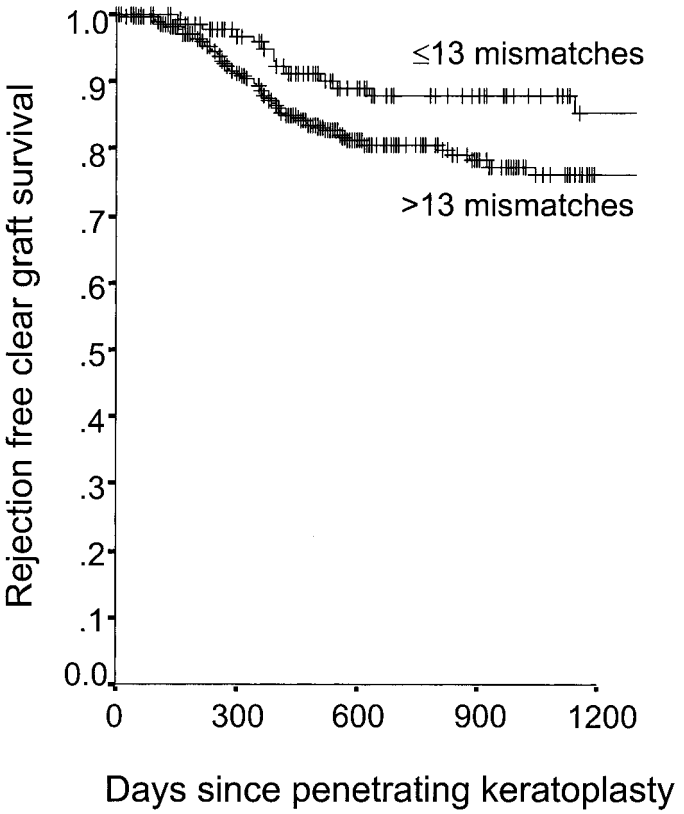

FIGURE 1. Triplet-string matching. Group I consists of the 147 keratoplasties with up to 13 mismatches at the triplet-string level. Group II consists of the remaining 398 less well-matched keratoplasties. Kaplan-Meier estimation: 85% vs. 76% rejection-free clear graft survival; log-rank test, $P=0.047$.

DISCUSSION

The HLAMatchmaker algorithm was originally designed and validated only to predict alloantibody formation in highly sensitized kidney graft recipients. Considering the role of cellular mechanisms in corneal graft rejection (*13*), applicability in penetrating keratoplasty is far from self-evident. Furthermore, assignment of triplets to HLA antigens in this study does lack precision because HLA typing for class I was performed by serologic methods that cannot differentiate molecular subtypes for which the triplet strings are defined exclusively: only DNA typing for HLA class I would permit deterministic accuracy in assignment of triples. Despite both potential drawbacks, a beneficial effect of the HLAMatchmaker algorithm in penetrating keratoplasty is demonstrated in this study.

This finding infers that the epitopes that elicit cellular and humoral immune responses, respectively, seem to be highly interrelated or even identical. Alternatively, the role of antibodies might currently be underestimated in penetrating keratoplasty (*19*, *20*).

Because of linkage disequilibrium and prospective HLA class I and II matching in the past, better HLA class I-matched groups are likely to be well matched for HLA-DR as well. A potential confounding factor for the positive effect from triplet-string matching is match grade at the HLA-DR locus (Tables 1–3), because it is known to be an influencing factor on rejection-free clear graft survival (*1*, *2*). The best way to rule out this confounding factor would be to include only keratoplasties with zero mismatches at HLA-DR in the triplet-string analysis. However, group sizes in this study resulting from this approach would prevent meaningful analysis (*21*). The beneficial effect of triplet-string matching in keratoplasties mismatched at the HLA-DR locus (Table 4) seems to be comparable to that of conventional HLA-A and HLA-B matching, where the percentage with good HLA-DR match grade is highest (Table 3). Thus, triplet-string matching seems to be mostly responsible for the observed reduction of graft rejections in this study but might be even higher in keratoplasties that are well matched with regard to HLA-DR status.

The unexpected inability to demonstrate any statistically significant HLA class I matching effect seems to be attributable to lack of statistical power. The log-rank test has poor statistical power when group sizes (group I) become small (*21*). The inability to demonstrate a statistically significant effect thus does not contradict recent studies (*1–3*).

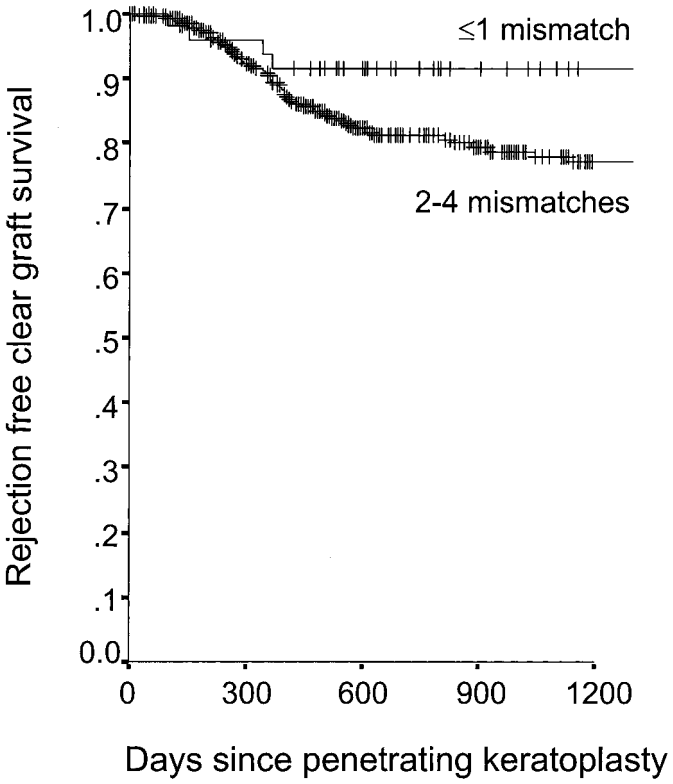

FIGURE 2. Conventional HLA-A and HLA-B matching. Group I consists of those 57 keratoplasties with up to one mismatch, and group II consists of the remaining 488 less well-matched keratoplasties. Kaplan-Meier estimation: 92% vs. 76% rejection-free clear graft survival; log-rank test, $P=0.08$.

Reduction of time on the waiting list resulting from triplet-string matching is substantial in most cases, paving the way for accepting as few mismatches at the triplet-string level as tolerable from the respective resulting waiting time. This strategy should be discussed with each patient individually. In addition, a good conventional HLA-DR match should be the goal, especially in high-risk cases (*1*). This approach will increase waiting time. When additional HLA loci (such as HLA-C and HLA-DQ) are considered in future matching strategies, the chance of finding suitable donors might further decrease despite triplet-string matching. Then, it might be useful to look for differential immunogenicity at the triplet-string level. Identification of acceptable triplet-string mismatches might help keeping time on the waiting list as short as possible. These analyses should include HLA types and rejection history of preceding keratoplasties and thus will have to be individual by nature.

CONCLUSION

Triplet-string matching seems to improve mid- to long-term prognosis in penetrating keratoplasty while reducing time on the waiting list in most cases. Alternatively, waiting time can be traded for a better match grade at high granularity on an individual basis. It should be considered for histocompatibility determination in penetrating keratoplasty after confirmation in a larger group with homogenous HLA-DR match grade.

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