Characteristics of Graft-Infiltrating Lymphocytes After Human Heart Transplantation

HLA Mismatches and the Cellular Immune Response Within the Transplanted Heart

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ABSTRACT The influence of HLA mismatches between donor and recipient on the phenotypes, function, and specificity of T-lymphocyte cultures derived from endomyocardial biopsies was studied in 118 heart transplant recipients In case of HLA-DR mismatches, the majority of the EMB-derived cultures were dominated by $CD4^+$ T cells while, in patients with HLA-A and -B mismatches but without DR mismatches, $CD8^+$ T cells comprised the predominant T-cell subset Cytotoxicity against donor antigens was observed in 75% of the cultures A significantly ($p < 0$ 005) lower proportion of the cultures showed cytotoxicity against HLA-A antigens (36%) when compared with HLA-B (53%) or HLA-DR (49%) An HLA-A2 mismatch elicited a cytotoxic response that was comparable to that found against HLA-B and -DR antigens 62% of the cultures from HLA-A2 mismatched donor–recipient combinations was reactive against A2 A higher number of A, B, or DR mismatches resulted in a higher number of cytotoxic cultures directed against these antigens A higher number of HLA-B and -DR mismatches was associated with a lower freedom from rejection Our data indicate that, despite the use of adequate immunosuppressive therapy, the degree of HLA matching plays a crucial role in the immune response against a transplanted heart, resulting in a significant effect on freedom from rejection *Human Immunology 39, 233–242 (1994)*

ABBREVIATIONS

CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T-lymphocyte precursor
EBV	Epstein–Barr virus
EMB	endomyocardial biopsy
MHC	major histocompatibility complex
PHA	phytohemagglutinin

INTRODUCTION

Products of the major histocompatibility complex (MHC) play a major role in the immune response against a transplanted organ [1, 2] In extensive studies in kidney transplant recipients, a positive effect of HLA matching on graft survival was reported, especially for HLA-B and -DR antigens [3–8] The beneficial effect of DR matching was found to be most evident in the first

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postoperative months, while the effect of matching for HLA-B antigens lasted longer These studies were based on the assumption that all of the HLA alleles had the same antigenic weight Busson and coworkers [9] showed, in kidney transplant recipients with only one incompatible HLA-antigen, that some of the HLA-A antigens were associated with a lower transplant survival than others, while at the B locus, there was no significant difference in survival rate among the different antigens

The importance of HLA matching for heart allograft survival is still debated, mainly because of the limited numbers of patients studied and, more importantly, the low numbers of well-matched grafts performed, as donor hearts are randomly allocated without reference to HLA matching Nevertheless, a beneficial effect of HLA matching has been found for cardiac graft survival [10, 11], the incidence of steroid-resistant rejection [12], or the freedom from rejection of the transplanted heart [13]

Acute allograft rejection is mediated by immunocompetent lymphocytes of the graft recipient that interact with allogeneic determinants expressed on the grafted organ Recognition of both HLA class I and class II allogeneic differences by both helper and cytotoxic T lymphocytes (CTLs) precipitates a cascade of reactions that results in a cytotoxic response directed against cells bearing these antigens, and thus in parenchymal damage of the graft tissue [14–20] In previous studies [21–25], we and other showed that graft-infiltrating lymphocytes can be cultured from cardiac graft tissue specimens During acute rejection episodes, a higher proportion of these biopsies yielded lymphocyte cultures, of which the majority was cytotoxic against donor-derived cells

The influence of HLA mismatches between donor and recipient on phenotypes and effector function of graft infiltrating cells has never been systematically studied Therefore we analyzed the effect of HLA-A, -B, and -DR mismatches on the functional and phenotypic characteristics of these cells in a large series of endomyocardial biopsies (EMBs) from 118 heart transplant recipients Moreover, we investigated the immunogenicity of individual mismatched HLA antigens

MATERIALS AND METHODS

Patients

We studied EMB-derived graft-infiltrating cells from 118 heart transplant recipients who underwent transplantations between February 1988 and January 1990 All patients had received preoperative blood transfusions and all received cyclosporine and low-dose prednisone as maintenance immunosuppression The actuarial patient survival was 89% at 4 years The mean number of mismatches between donor and recipient was 1 25, 1 62, and 1 40 for HLA-A, -B, and -DR, respectively Table 1 shows the distribution of the patients among the different matched groups HLA histocompatibility was based on matching for broad specificities Homozygosities were considered as one mismatched antigen

Rejection was monitored by EMB Grading of the biopsies was according to Billinghams [26] criteria of none, mild, moderate, and severe rejection For the diagnosis of moderate rejection, the coexistence of myocyte necrosis and mononuclear infiltrates was required In that case, antirejection treatment was instituted, which consisted of bolus steroids or, in case of ongoing rejection, of a 2-week course of a polyclonal rabbit antithymocyte-globulin preparation There were no cases of severe rejection In the early posttransplant period, serial biopsy specimens were obtained at weekly intervals Later, EMBs were taken less frequently, declining to once very 4 months at 1 year After an acute rejection episode, the next biopsy sample was taken 1 week following rejection therapy Three patients who died within 3 weeks after transplantation (from other causes than severe rejection) were excluded from this study We received a total of 1285 EMBs, 4–22 from each patient (median, 10)

HLA Typing

Spleen cells or peripheral blood mononuclear cells (obtained by Ficoll separation of heparinized blood) were typed for HLA class I antigens according to the standard National Institutes of Health lymphocytotoxicity assay, and typed for HLA-DR by the two-color fluorescence assay with a set of highly selected antisera [27]

Culture Method

Lymphocyte cultures These were established from EMBs as described previously [21] In brief, each biopsy specimen was divided into two or more fragments and placed into two or more wells of a 96-well round-bottom tissue culture plate (Costar 3799, Cambridge, MA, USA) with 200-μl culture medium in the presence of 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells as

TABLE 1 Distribution of the 118 patients among the different mismatched groups

	Number of patients		
HLA	0 MM[a]	1 MM	2 MM
A	11	67	40
B	4	40	74
DR	9	53	56

[a] MM, mismatch

feeders Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% vol/vol lectin-free Lymphocult-T-LF (Biotest, Dreieich, Germany) as exogenous source of IL-2, 10% pooled human serum, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin When growth was observed, the contents of several wells of a culture were pooled and transferred to more wells when sufficient cell density was reached (10^5–10^6 cells/ml) When growth was slowing down or cell death was observed, the cultures were restimulated by adding either 10^5 irradiated (40 Gy) donor spleen cells/well or 5×10^3 Epstein–Barr virus (EBV)-transformed donor cells/well (irradiated with 80 Gy) Only 6% of the cultures needed restimulation in order to obtain sufficient amounts of cells for a cell-mediated cytotoxicity assay

Allogeneic stimulator/target cells Phytohemagglutinin (PHA) blasts were obtained by culturing spleen cells for at least 5 days in the presence of 1% PHA-M (Difco, Detroit, MI, USA) and culture medium RPMI 1640 +5% pooled human serum and 5% lymphocult-T (Biotest) EBV-transformed B-cell lines were set up and cultured as previously described [28]

Phenotypic Analysis

The phenotypes of the graft-infiltrating lymphocytes were analyzed by two-color flow cytometry after staining with monoclonal antibodies directed against CD8 (anti-leu2) and CD4 (anti-leu3), both purchased from Becton Dickinson (Mountain View, CA, USA) The antibodies were directly conjugated to fluorescein or phycoerythrin A more extensive phenotypic characterization of the cultured cells is described elsewhere [21] A T-cell subset was considered to be predominant when it comprised more than 60% of the cells in a culture

Cell-Mediated Cytotoxicity Assays

Biopsy-derived bulk cultures were tested for donor-directed cytotoxicity in a standard 4-hour ^{51}Cr-release assay according to the European Standard Technique [29] As target cells we used donor-derived cell lines and a panel of unrelated target cells (PHA T-cell blasts or EBV-transformed B-cell lines) sharing one or more HLA antigens with the donor, and a third-party control The specificity for donor HLA class I (HLA-A and -B) or class II (HLA-DR) antigens was determined by testing the cytotoxicity of the biopsy-derived T-cell lines against a panel consisting of 5–10 (median, 7) target cell lines Each individual HLA antigen was represented 1–4 times in the cell panels If the cytotoxicity against an HLA antigen was difficult to interpret, such as the example of HLA-B15 in Fig 1 (left panel), this antigen was considered to be not tested The HLA antigens studied in the present report are listed in Table 2

^{51}Cr-labeled target cells, $2\,5 \times 10^3$, were mixed with effector cells in 200 μl culture medium per well in 96-well U-bottom microtiter plates (Costar) Serial double dilutions with effector–target ratios varying from 1 25 1 up to 80 1 were used The plates were incubated for 4 hours at 37°C in 5% CO_2 Supernatants were harvested with a Skatron harvesting system (Skatron-AS, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard Instruments, Downers Grove, USA) According to the recommendations of the European CML Workshop [29], cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector–target ratio of 20 1 or greater, and the slope of a graph was positive Some representative cytotoxicity titrations are represented in Fig 1 Series of double-dilution studies revealed that lysis percentages of autologous control cell lines did not exceed 10%

Statistical Analysis

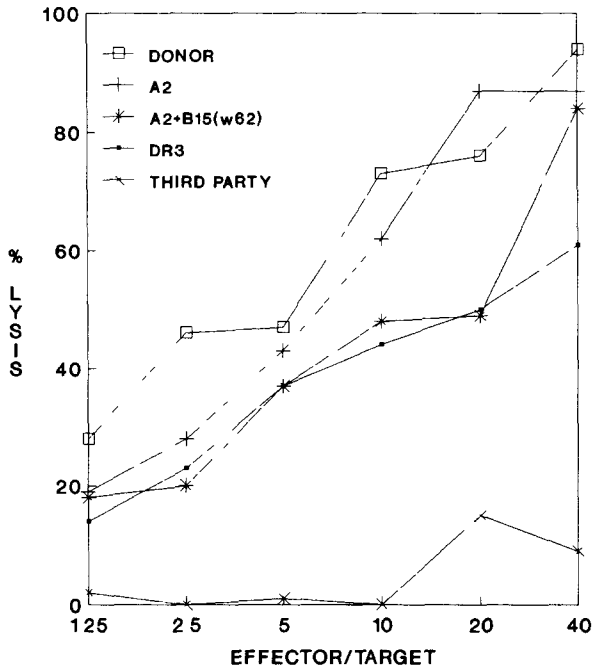
The significance of differences among the various groups of patients and cultures was analyzed by Mann–Whitney test or by chi-squared test, respectively Freedom from rejection rates were computed by actuarial methods and statistical significance was estimated by log-rank analysis

RESULTS

HLA mismatches and acute rejection In the DR-matched patient group 56% of patients remained free from rejection at 6 months, compared with 29% of patients with one and 22% with two DR mismatches with their donors For the combination of HLA-B and -DR antigens a significant effect on freedom of rejection was observed ($p < 0\ 05$, Fig 2) Transplants with two or fewer mismatched HLA-B and -DR antigens displayed a 14% higher freedom from rejection at 6 months compared with those with three or four HLA-B and -DR incompatibilities The number of HLA-A mismatches did not show any additive effect on freedom from rejection rates

No significant relation between the number of acute rejection episodes and the number of mismatches on the individual A, B, or DR locus was observed

HLA mismatches and CD4/CD8 phenotypes From all patients, approximately 60% of the EMBs yielded lymphocyte cultures The success of culturing did not depend on the degree of HLA-DR matching between donor and recipient, since the mean percentages growing biopsy specimens were highly comparable in patients with zero, one, or two DR mismatches with their donors (57%,



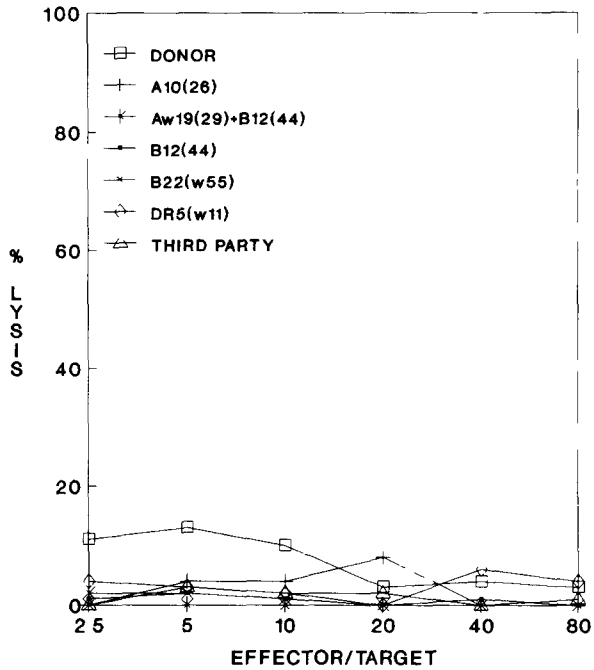


FIGURE 1 Representative example of a positive (left) and a negative cytotoxicity titration (right) of biopsy-derived cultures from two different patients The cultures were not restimulated with donor cells and were cultured for 40 and 26 days, respectively In these examples, EBV-transformed B-cell lines were used as targets The HLA antigens shared by target cells and the heart donor are indicated HLA typing (left) recipient A1, A10(25), B18, B35, DR2(w15), and DRw6(w13), and donor A2, B15(w62), B18, DR2(w15), and DR3, and (right) recipient A2, A3, B35, B37, DR7, and DRw10, and donor A10(26), A19(29), B12(44), B22(55), DR5(11), and DR7

59%, and 63%, respectively) In the first 180 days after transplantation, the number of HLA-DR mismatches between donor and recipient had a pronounced influence on the phenotypic composition of the EMB-derived lymphocyte cultures (Fig 3) Recipients with HLA-A and -B mismatches but without DR mismatches with their donors yielded cultures that were dominated by $CD8^+$ T cells in 60% of cases ($p < 0\ 005$ compared with DR-mismatched combinations) In cultures from patients with DR mismatches, $CD4^+$ T cells comprised the predominant T-cell subset The predominance of $CD4^+$ T cells was most evident in patients with two DR mismatches with their donors ($p = 0\ 025$ compared with DR-matched combinations) After the first 6 posttransplant months, no significant differences between the groups were found

The influence of the degree of HLA class I matching on growth and phenotypic composition of the cultures was hard to evaluate, because all patients except one had one or more class I mismatches The EMB-derived cultures ($n = 7$) from this single patient were all dominated by $CD4^+$ T cells In the remaining patients, no significant relation was found between the number of HLA class I mismatches and the degree of CD8 predominance

Cytotoxicity In vitro studies showed that the majority ($n = 234$ or 75%) of the tested EMB-derived T-lymphocyte cultures ($n = 324$) was cytotoxic against donor antigens Of these cultures, 165 (53%) and 154 (49%) were cytotoxic against HLA-B and -DR antigens, respectively Significantly fewer cultures ($n = 107$, 36%, $p < 0\ 005$) showed cytotoxicity against HLA-A antigens This higher immunogenicity of HLA-B and -DR antigens was also apparent when we analyzed the CTL reactivity against several individual HLA antigens (Table 2) This was analyzed for broad specificities and not splits Against most HLA-A antigens a low percentage of reactive cultures was found Only HLA-A2 was found to be of comparable immunogenicity to HLA-B and -DR antigens Of the 90 tested cultures from 23 donor–recipient combinations with an HLA-A2 incompatibility, 62% showed reactivity against A2, which was significantly higher than generally found against other HLA-A antigens ($p < 0\ 001$, Table 2) When analyzed as the percentage reactive cultures for each individual patient, a median of 60% HLA-A2-reactive cultures was found, which was again significantly higher than against other mismatched HLA-A antigens ($p < 0\ 001$, Fig 4) Among the HLA-B antigens, no evidence for such an immunodominant locus allele was found Generally, a high percentage of tested cultures showed cytotoxicity against mismatched HLA-B antigens, while against some of these antigens the reactivity was lower (Tables 2 and 3) A similar pattern was found for reactivity against HLA-DR mismatches (Table 3)

TABLE 2 CML reactivity of EMB-derived lymphocyte cultures against the most prevailing mismatched HLA antigens, expressed as numbers and percentages of cultures reactive against an HLA antigen

HLA MM[a]	Number of cultures: Tested	Positive in CML (%)	No of patients tested
HLA-A			
A1	73	18 (25)	24
A2	90	56 (62)	23
A3	58	19 (33)	22
A10	38	7 (18)	12
A11	36	7 (19)	7
Aw19	119	32 (27)	29
HLA-B			
B5	58	23 (40)	11
B7	46	21 (46)	19
B8	54	23 (43)	14
B12	68	38 (56)	19
B18	34	13 (38)	11
B27	21	11 (52)	7
B35	59	32 (54)	15
B40	37	9 (24)	15
B15	30	4 (13)	9
HLA-DR			
DR1	85	35 (41)	20
DR2	42	17 (40)	16
DR3	62	31 (50)	19
DR4	72	33 (46)	17
DR5	87	32 (37)	18
DRw6	50	22 (44)	12
DR7	59	29 (49)	16
DRw8	37	10 (27)	10

[a] MM, mismatch

In time, donor-directed cytotoxicity declined significantly ($p < 0.03$), from 79% (181 of 229) of the cultures established from EMB taken in the first 180 days to 61% (58 of 95) after 6 months This was due to a fall in the percentage of cultures that were cytotoxic against mismatched donor HLA-B and -DR antigens (Table 4)

A higher number of HLA-A, -B, or -DR mismatches between donor and recipient was found to be positively correlated with the percentage of cytotoxic cultures directed against these antigens (Table 5) Biopsy samples from patients with two A, B, or DR mismatches with their donors yielded a higher proportion of cytotoxic cultures directed against these antigens when compared with EMB from patients who had only one mismatch for any of these HLA antigens The pronounced dose effect of HLA-A mismatches was mainly found when the second HLA-A antigen was A2 (in 62% of cultures from donor–recipient combinations with two HLA-A mismatches and a positive CML against HLA-A, compared with only 28% of cultures not reactive against HLA-A, $p < 0.001$, χ^2 test)

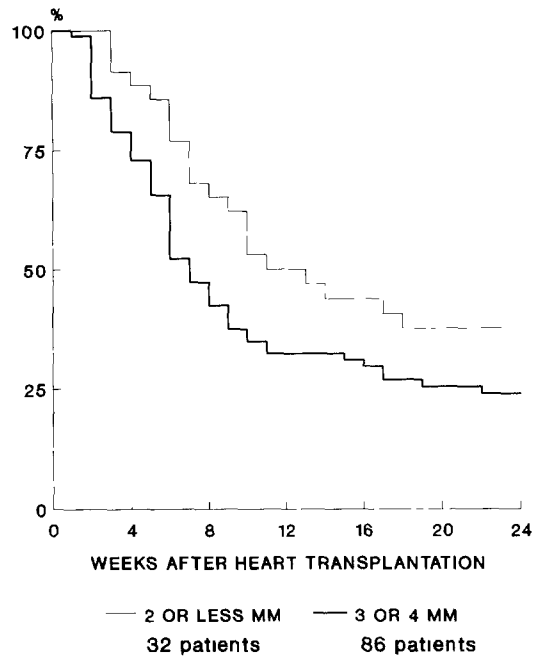


FIGURE 2 Actuarial freedom from rejection of heart transplants in relation to matching for HLA-B and -DR antigens The freedom from rejection of patients with ≤2 or >3 mismatches for the combined B and DR antigens were 65% and 42% at 2 months ($p = 0.01$), and 37% and 24% at 6 months ($p = 0.05$), respectively

DISCUSSION

The present study shows that the number of mismatched HLA-B and -DR antigens on a transplanted heart, but also HLA-A antigens, is positively correlated with the percentage of cytotoxic EMB-derived cultures directed against these mismatched HLA antigens The incidence of HLA-A-directed cytotoxicity was lower, however, than that directed against B or DR mismatches This apparently high immunogenicity of HLA-B and -DR antigens may account for the significantly lower freedom from rejection rates in the patient group with more than two HLA-B and -DR mismatches This association between the number of HLA-B and -DR mismatches and freedom from rejection has also been described by others [13] Studies on the effect of matching for HLA antigens in renal [3–8] and heart transplantation [10, 11] showed that matching for HLA-B and -DR has a significant influence on graft survival In normal individuals HLA-B antigens are more immunogenic to cytotoxic T cells than HLA-A antigens, although major individual differences were found in the frequency of alloreactive CTL precursor (CTLp) directed against HLA class I [30–33] and class II [34] antigens Until now, a direct cor-

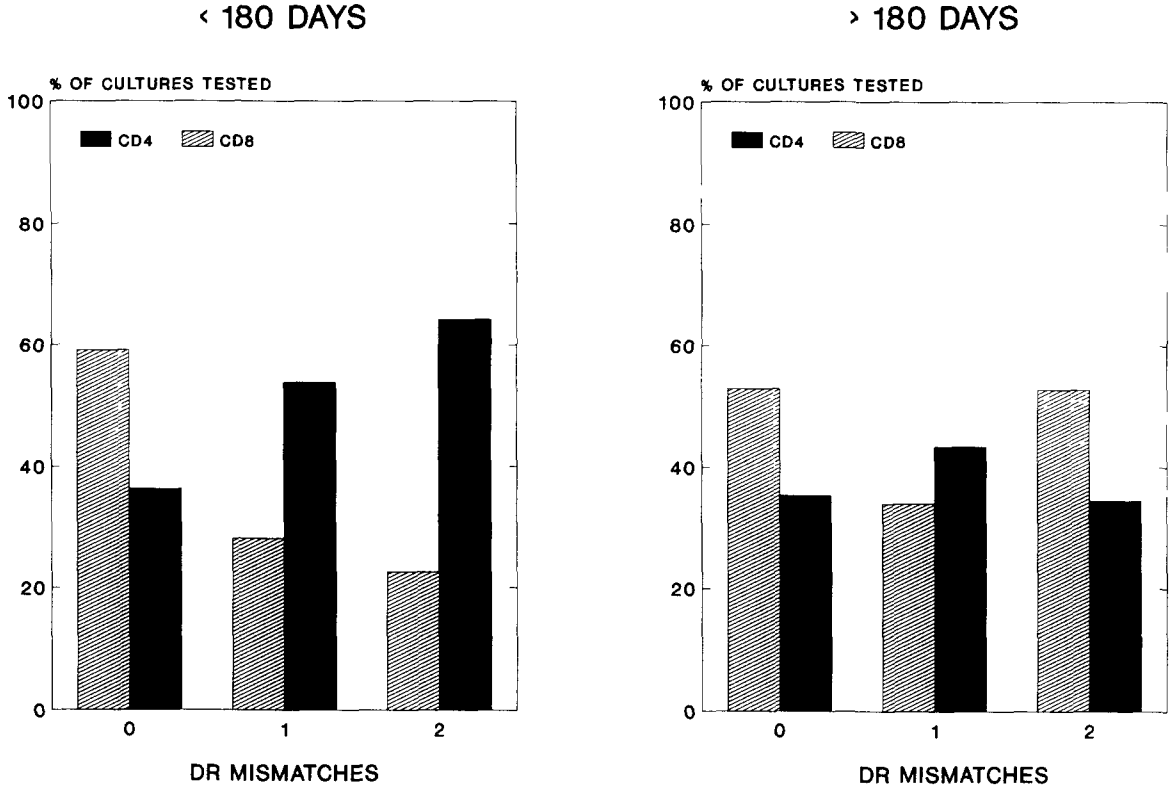


FIGURE 3 Predominant phenotype of EMB-derived lymphocyte cultures in relation to the number of HLA-DR mismatches (MM) between donor and recipient In the first 180 days after transplantation, cultures from patients without DR mismatches with their donors were most often dominated by $CD8^+$ cells ($p < 0\ 005$ compared with DR-matched combinations), while in cultures from patients with DR mismatches $CD4^+$ T cells comprised the predominant subset in the majority of cultures **(left)** After the first 6 posttransplant months, no significant differences between the groups were found **(right)** Numbers of cultures tested **(left)** 0 DR MM ($n = 22$), 1 DR MM ($n = 160$), and 2 DR MM ($n = 226$), and **(right)** 0 DR MM ($n = 17$), 1 DR MM ($n = 106$), and 2 DR MM ($n = 110$)

relation between pretransplant CTLp frequencies and transplant outcome has not been shown But it was found in mice that an increase of CTLp frequency after transplantation is associated with allograft rejection [35] In renal transplant patients, a decrease of donor-specific CTLp is correlated with good graft function [36], and a low patient-specific CTLp frequency in a bone marrow donor gives less graft-versus-host disease [37]

In the present study, a "dose–effect" phenomenon of the number of mismatches on CML reactivity was found for HLA-A, -B, and -DR antigens This dose–effect phenomenon of HLA mismatches could also be observed in the higher 6-month freedom from rejection in patients with two or fewer HLA-B and -DR mismatches in the first half year This is consistent with data of Opelz [3, 10, 38], who showed that during the early posttransplant course, HLA-B and -DR mismatches exerted a strong influence on transplant survival, in contrast to mismatched HLA-A antigens However, the influence of the latter on long-term survival was comparable to that of HLA-B and -DR antigens [38] Interestingly, the dose effect for HLA-A mismatches was mainly found when the second mismatched antigen on the A locus was A2

Our finding of high reactivity against HLA-A2 antigens expressed on donor cells confirms the observation of others [33] (Roelen, personal communication) that HLA-A2 is an immunodominant locus allele They found high anti-A2 CTLp frequencies among normal individuals and in highly sensitized patients awaiting renal transplantation, respectively By inhibition experiments with CD8 monoclonal antibodies, it was shown that all anti-HLA-A (including A2) CTLps could be inhibited, indicating that these cytotoxic T cells have a low avidity for HLA-A antigens Of HLA-B-directed CTLs, significantly fewer could be inhibited In previous reports [39, 40], we have shown that low-avidity alloreactive CTLs are probably not relevant for the rejection process High-avidity alloreactive CTLs can be demonstrated in the peripheral blood of highly sensitized candidates for renal (re)transplantation, and in the grafts of rejecting heart transplant recipients Interestingly, preliminary data from Eurotransplant (Thorogood) show

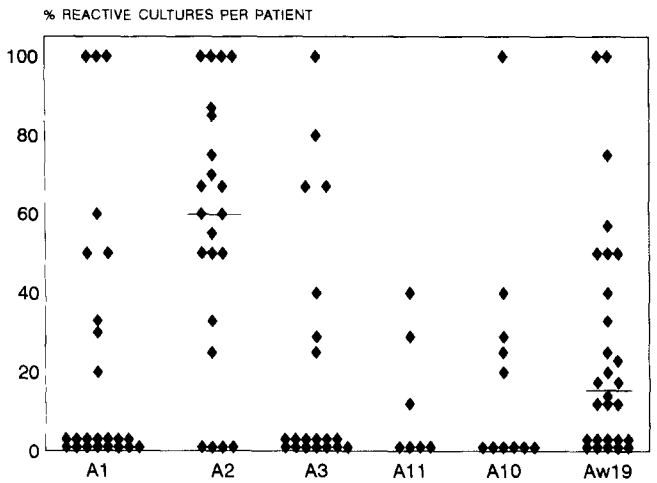


FIGURE 4 Percentage cytotoxic cultures against individual mismatched HLA-A antigens *Each dot* represents one patient From each patient, 1–12 (median, 4) biopsy-derived cultures could be tested Against an HLA-A2 mismatch, a significantly higher percentage of reactive cultures was found when compared with other mismatched HLA-A antigens ($p < 0.001$, Mann–Whitney test) Medians of a group are indicated as *horizontal lines*

TABLE 3 CML reactivity against mismatched HLA-B and -DR antigens, expressed as median percentage reactive cultures per patient group with this HLA incompatibility

	Median percentage reactive cultures
HLA-B	
B5	22
B7	50
B8	38
B12	60
B18	60
B27	50
B35	60
B40	0
B15	0
HLA-DR	
DR1	45
DR2	23
DR3	40
DR4	67
DR5	33
DRw6	22
DR7	40
DRw8	6

From each patient, 1–12 (median, 4) biopsy-derived cultures could be tested

TABLE 4 CML specificity of EMB-derived cultures against panel cells sharing mismatched HLA-A, -B, or -DR antigens with the donor relation with time after transplantation

	Number of reactive cultures		
CML specificity	<180 days *n* (%)[a]	>180 days *n* (%)	p^b
HLA-A	79 (38)	28 (33)	NS
HLA-B	127 (57)	38 (40)	<0 01
HLA-DR	120 (55)	34 (37)	<0 01

In the first half-year, the incidence of HLA-A-directed cytotoxicity was significantly lower than that against B or DR antigens ($p < 0.001$)

[a] Number and percent of reactive cultures

[b] χ^2 test

that a mismatch for HLA-A2 does not result in a lower kidney transplant survival In the present study, this could not be evaluated, because all patients also had several HLA-B and -DR mismatches with their donors

In the one and two DR-mismatched groups, significantly more CD4-dominated cultures were derived from the biopsies than in the DR-matched group $CD4^+$ cells are known to be of crucial importance in the initiation of rejection [14–20] Interaction of these cells with donor class II MHC antigens expressed on the graft tissue and on passenger leukocytes of donor origin results in activation of $CD8^+$ cells that recognize MHC class I antigens Both $CD4^+$ and $CD8^+$ cells play a role in the rejection of mismatched grafts [14–16] Rejection of class I disparate grafts appears to depend most on $CD8^+$ cells, although $CD4^+$ cells can be activated as well via presentation of donor MHC class I antigens on recipient antigen-presenting cells in the context of self-HLA class II molecules [41, 42]

More than 180 days after transplantation, the decline found in the number of the CD4-dominated cultures may be due to a lower expression of donor-type class II antigens on graft tissue, due to the replacement of donor dendritic cells by the patients' antigen-presenting cells [43, 44] As a consequence, fewer class-II-specific $CD4^+$ lymphocytes may be attracted to the graft Data from animal heart transplant models show that this reduction of the number of HLA class-II-expressing dendritic cells may already start early after transplantation, which may explain our finding that in some patients an earlier decline of DR-directed cytotoxicity is found [45] (data not shown)

Lower expression of donor-type HLA antigens on graft tissue may also play a role in the lower incidence of cytotoxicity directed against these antigens after 6

TABLE 5 The dose effect of the number of HLA mismatches per locus on CML reactivity of EMB-derived cultures

CML specificity	Number of reactive cultures: 1 MM[a] *n* (%)[b]	2 MM *n* (%)	*p*[c]
HLA-A	86 (34)	77 (52)	0 005
HLA-B	78 (50)	145 (60)	0 055
HLA-DR	73 (40)	119 (52)	0 025

[a] MM, mismatch

[b] Number and percent of reactive cultures

[c] χ^2 test

months [47, 48] Next to the influence of HLA expression on the graft, other mechanisms may also be involved Specific suppression of antidonor responses by regulatory T cells, clonal anergy or deletion of antidonor T cells, or downmodulation by antiidiotypic antibodies are thought to be important mechanisms contributing to acquired immune tolerance in human transplant recipients [21, 36, 48–50]

In conclusion, we showed that the number and nature of HLA mismatches between donor and recipient strongly influence the cellular immune response within the transplanted heart, resulting in a significant effect on freedom from rejection

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