Alloreactive Lymphoid Infiltrates in Human Heart Transplants
Loss of Class II-Directed Cytotoxicity More Than 3 Months After Transplantation

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ABSTRACT: From 535 endomyocardial biopsies (87 heart transplant recipients) 283 cell cultures could be generated. All cultures tested contained T lymphocytes and in most cases CD4 was the predominant phenotype at any time posttransplant. A significantly higher proportion of CD8-dominated cultures was found among cultures from biopsies without myocytolysis.

In the first 3 months post transplant 57% of cultures showed cytotoxicity against both class I and class II mismatched donor major histocompatibility complex (MHC) antigens, changing to an incidence of 33% at > 90 days. This proved to be due to a significant decrease in the number of cultures with human leukocyte antigen class II-directed cytotoxicity.

This study shows that early after transplantation a heart transplant is infiltrated with activated donor-specific cytotoxic T cells which recognize a broad spectrum of mismatched donor MHC antigens, and that in time this spectrum becomes more restricted. *Human Immunology 30, 50–9 (1991)*

ABBREVIATIONS

B-LCL	B-lymphoblastoid cell line
CML	cell-mediated lympholysis
CTL	cytolytic T lymphocytes
EMB	endomyocardial biopsy
EBV	Epstein-Barr virus
FACScan	fluorescence-activated cell scan
HLA	human leukocyte antigen
IL-2	interleukin-2
MHC	major histocompatibility complex
NK	natural killer
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
TCR	T-cell receptor

INTRODUCTION

The diagnosis of rejection after clinical heart transplantation is based on histologic criteria [1]. Therefore endomyocardial biopsies (EMB) are taken at regular intervals after transplantation. This provides us with the opportunity to culture graft- infiltrating cells which makes it possible to evaluate growth patterns, phenotypic composition, and function of these cells both in periods of graft stability and during rejection. The biopsies were cultured in interleukin-2 (IL-2)-conditioned culture medium in the presence of irradiated autologous peripheral blood mononuclear cells (PBMC) as feeder cells, thereby assuming that only in vivo-activated lymphocytes will proliferate [2, 3]. Neither donor nor third-party cells were added within the first 3 weeks of culture to avoid in vitro activation. In the present re-

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port, we describe the phenotypic and functional characteristics of lymphocytes grown from EMB, and the relation of these findings with time after transplantation and histopathological diagnosis.

METHODS

Patients. We studied 535 biopsies from 87 heart transplant recipients transplanted between January 1985 and February 1989. During this transversal study we received 2 to 18 EMB from each patient (median 4). EMB were taken 5 to 1587 days (median 114) after transplantation. All patients had received preoperative blood transfusions and all received cyclosporine and low-dose prednisone only as maintenance immunosuppression. Their actuarial graft survival at 3 years was 89%. Acute rejection episodes, i.e., biopsy-proved myocytolysis, were treated with bolus steroids or with a 2-week course of a polyclonal rabbit antithymocyte globulin preparation in case of ongoing rejection. In the early posttransplant period serial biopsies were obtained at weekly intervals. Later EMB were taken less frequently, declining to once every 4 months at 1 year. After an acute rejection episode the next biopsy was taken 1 week following rejection therapy. During right ventricular catheterization four or five biopsy samples were obtained. Three or four samples were used for histological evaluation, and one was placed in RPMI-1640 for tissue culture. The histological rejection grade was assessed according to Billingham's criteria [1]: grade 0: no evidence of rejection, no infiltrate; grade 1: mild infiltration of mononuclear cells, endocardial and interstitial edema, diffuse perivascular and endocardial infiltration with pyroninophilic lymphocytes; grade 2: moderate rejection, perivascular, endocardial, and interstitial infiltrates with pyroninophilic lymphocytes, focal myocytolysis (necrosis); grade 3: severe rejection, vessel wall and myocyte necrosis with interstitial bleeding, interstitial infiltrates with polymorphonuclear cells and pyroninophilic lymphocytes (this rejection grade was not observed in our study); grade 4: resolving rejection, active fibrosis, some small nonpyroninophilic lymphocytes, some plasma cells, and hemosiderin.

Culture method. Each biopsy was divided into two or more fragments and placed into two or more wells of a 96-well roundbottom tissue culture plate (Costar 3799, Cambridge, MA) with 200 μl culture medium in the presence of 10^5 irradiated (40 Gy) autologous PBMC as feeders. Culture medium consisted of RPMI-1640-Dutch modification (Gibco, Paisley, Scotland) supplemented with 10% vol/vol lectin-free Lymphocult-T-LF (Biotest GmbH, Dreieich, FRG) as exogenous source of IL-2, 10% pooled human serum, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. PBMC were isolated by Ficoll-Isopaque (σ = 1.077) density-gradient centrifugation. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Half the culture medium was refreshed every 2 to 3 days. 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 to 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, when available, 10^4 Ebstein-Barr virus (EBV)-transformed donor cells/well (irradiated with 80 Gy). This was not done in the first 3 weeks of culture.

B-lymphoblastoid cell lines (B-LCL) originated from infection of fresh PBMC or spleen cells with EBV obtained from the marmoset cell line B95-8 as described by Moreau et al. [4]. These cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, England).

T-lymphoblastoid cell lines were obtained by culturing PBMC or spleen cells in RPMI 1640 supplemented with 5% pooled human serum, 5% vol/vol Lymphocult-T (Biotest), and 1% phytohemagglutinin (PHA).

K562 was cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum.

Phenotypic analysis. Surface differentiation antigens were analyzed by two-color flow cytometry after staining with monoclonal antibodies directed against CD3 (anti-leu4) as a pan mature T-cell marker, WT31 as a marker for the α/β chain of the T-cell receptor (TCR), and CD8 (anti-leu2) and CD4 (anti-leu3) as T-cell subset markers. CD16 (anti-leu 11) and CD56 (anti-leu 19) were used as markers for natural killer (NK) cells. Antibodies were directly conjugated to fluoresceine (FITC) or phycoerythrin (PE) (Becton & Dickinson, Mountain View, CA). The presence of γ/δ T cells was demonstrated by the monoclonal anti-TCR-γ/δ-1 (clone 11F2) [5] by an indirect fluorescence technique. Cells were stained by incubating 0.5 to 1 × 10^5 cells in 50 μl Hanks balanced salt solution (Biochrom KG, Berlin) supplemented with 1% bovine serum albumin and 0.1% sodiumazide for 30 min at room temperature with two antibodies conjugated to different fluorochromes. After washing, cells were analyzed on a fluorescence-activated cell scan (FACScan) flow-cytometer (Becton and Dickinson).

Cell-mediated cytotoxicity. Cytotoxicity was tested against donor cells and a panel of unrelated target cells sharing one or more human leukocyte antigens (HLA) with the donor. Fifty-nine bulk cultures could be tested

before restimulation with allogeneic cells. The remaining cultures had to be restimulated in order to obtain sufficient numbers of cells. No effect of restimulation on cell-mediated lympholysis (CML) specificity could be demonstrated after repeated testing. Three types of target cells were used: PHA-blasts (HLA class I targets), B-LCL (class I and II targets), and the K562 cell line for assessment of NK cell activity. A standard 4-hr ^{51}Cr-release assay was performed [6] with one effector-target ratio of 20:1. When possible E:T ratios varying from 1.25:1 up to 80:1 were used. Target cells were incubated for 1½ hr at 37°C with 200 μCi ^{51}Cr (Na_2CrO_4 5 mCi/ml, specific activity 350–600 mCi/mg chromium, Amersham, UK). ^{51}Cr-labeled target cells (2.5×10^3) were mixed with effector cells in 0.2 ml of culture medium in 96-well roundbottom tissue culture plates. The plates were centrifuged at 600 g for 1 min, incubated at 37°C, and supernatants were collected after 4 hr (Skatron AS, Lier, Norway). Spontaneous chromium release was determined by incubation of targets in 0.2 ml culture medium, and maximum release was obtained by adding 10% Triton X-100 detergent (5% vol/vol solution in 0.01 TRIS buffer) to the targets. Experimental release was measured and specific lysis was calculated with the following equation:

$$\text{Specific lysis} = 100 \times \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}$$

A CML assay was considered positive when the percentage specific lysis of donor antigen-bearing target cells exceeded 10% and the slope of a graph was positive [6]. Series of double dilution studies revealed that lysis percentages of less than 10% are within the variation range of the assay (data not shown).

For statistical analysis of all data a chi-square test, with Yates correction when appropriate, was performed.

RESULTS

Generation of lymphocyte cultures. In total 283 lymphocyte cultures were established from 535 EMB (53%). From the majority of patients (72/87) cells could be successfully grown from at least one biopsy. From the remaining 15 patients only a few biopsies were available and these were almost always taken more than 1 year after transplantation. Cell growth was most successful from EMB taken in the second and third postoperative months (Table 1). After 3 months a significant decrease of the growth percentage was observed ($p < 0.005$ when compared to all EMB taken in the first 3 months). Table 2 shows that the rate of establishing cultures is positively correlated with increasing histological rejection grade. Even when no mononuclear cells were detected by histology (grade 0 or 4) cell growth was obtained in 37% of the cases. These grade 0/4 biopsies showed the highest growth percentages between 11 and 180 days after transplantation with a peak of 64% in the second and third postoperative months and a decline to 33% after 180 days ($p < 0.01$ when the period between 11 and 180 days is compared with > 180 days). EMB showing histological signs of infiltrate (grades 1 and 2) yielded more cultures than grade 0/4 biopsies independent of the time after transplantation. After the first 90 days a decline of the growth rate of grade 1 EMB was observed ($p < 0.01$). Grade 2 EMB always showed high growth percentages (Table 2).

Phenotypic analysis. Flow cytometric analysis of cell surface molecules of 200 cultures showed that the majority

TABLE 1 Culture results and numbers of cultures analyzed for phenotype and cytotoxicity in relation to time after transplantation

Days after transplantation	No. patients	No. EMB				
		Obtained	Growing	(%)	Analyzed for phenotype	Tested in CML
0–10	30	31	7	(23)	6	4
11–30	31	80	46	(58)	39	24
31–90	32	125	91	(73)	74	49
91–180	34	96	55	(57)	36	25
181–365	32	83	31	(37)	21	7
>365	50	120	53	(44)	24	17
Total	87	535	283	(53)	200	126

TABLE 2 Relationship of successful cell growth from EMB with histological rejection grades (Billingham's criteria) in different time intervals after transplantation

Days post transplant	Grade 0, 4[a]			Grade 1			Grade 2		
	No. EMB	Growing	(%)	No. EMB	Growing	(%)	No. EMB	Growing	(%)
0–10	26	3	(12)	5	4	(80)	0		
11–30	25	10	(40)	46	30	(65)	9	6	(67)
31–90	25	16	(64)	83	60	(72)	17	15	(88)
91–180	26	14	(54)	59	32	(54)	11	9	(82)
181–365	42	9	(21)	41	22	(54)	0		
>365	75	29	(39)	44	23	(52)	1	1	
Total EMB	219	81	(37)	278	171	(62)	38	31	(82)
No. patients	78	52		64	53		23	20	

[a] Grades 0 and 4 EMB were grouped together because these show equal growth percentages.

(89.5%) exclusively consisted of cells carrying the CD3 determinant. Almost all $CD3^+$ cells were $WT31^+$. The CD4/CD8 makeup of the T cells in 200 cultures is shown in Table 3. Twenty-nine cultures contained only $CD4^+$ T cells and 15 consisted of $CD8^+$ cells only. In three cases exclusively $CD4^-CD8^-$ cells were found (TCR τ/δ^+ cells, described below). All except three recipients whose EMB cultures could be tested for phenotype had multiple mismatches for HLA class I and II with their donors. The cultures from one patient with only one class II and no class I mismatches contained only $CD4^+$ cells (three EMB). Five EMB from two other patients of whom one had one mismatch on the B locus and the other an additional mismatch on the A locus yielded pure CD8 cultures as well as mixed cultures. One culture even contained $CD4^+$ T cells only. No significant difference in the number of mismatches between donor and recipient could be demonstrated between patients supplying pure CD8 or pure CD4 cultures (mean number of mismatches: 2.80 versus 2.85 for HLA A,B and 1.60 versus 1.45 for HLA DR, respectively). In the remaining 153 cultures both $CD4^+$ and $CD8^+$ cells were found and the greatest fraction comprised cultures with CD4 as the predominant phenotype at any time after transplantation. Again, no significant relation with the number of mismatches was found. In the cultures originating from biopsies with myocytolysis the preponderance of CD4 cells was more striking and a significantly lower proportion of CD8-dominated cultures was found (Fig. 1, $p < 0.025$).

In 16 cultures from 13 patients $CD3^+WT31^-11F2^+$ cells (TCR τ/δ^+) were found in amounts varying from 5% to 100% of the cultured cells (median 27%). The EMB were taken between 29 and 1324 days posttransplant (median 624 days). In four cases (four different patients) the $\tau\delta^+$ cells dimly expressed CD8. The remaining cultures only contained $CD4^-$ $CD8^-$ $\tau\delta^+$ cells. We found $\tau\delta^+$ cells significantly more often at more than 1 year after transplantation (11/24 versus 5/176 phenotyped cultures, $p < 0.001$). Three of four dimly

TABLE 3 Predominant phenotype of 200 cultures (59 patients) in relation to time after transplantation

Days posttransplant	Mixed cultures (*n*)				Single-cell-type cultures (*n*)		
	>60% CD4	CD4 = CD8	>60% CD8	>60% $CD4^-CD8^-$	Only CD4	Only CD8	Only $CD4^-CD8^-$
0–30	18	13	8	0	3	3	0
31–90	25	15	19	0	12	3	0
91–180	10	5	6	1	11	3	0
>180	17	5	9	2	3	6	3
Total EMB	70	38	42	3	29	15	3
No. patients	42	25	28	3	20	15	3

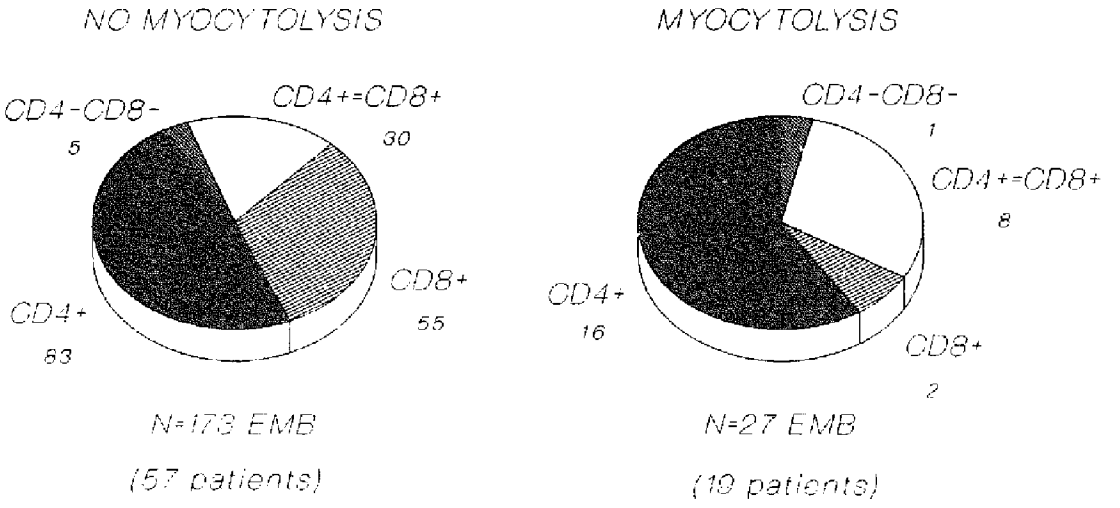

FIGURE 1 Proportion of cultures dominated by (>60%) $CD4^+$, $CD8^+$, or $CD4^-8^-$ cells or containing equal amounts of $CD4^+$ and $CD8^+$ cells. The phenotypic composition of 173 cultures derived from EMB without myocytolysis (histology grades 0 and 1) was compared with that of 27 cultures from EMB with myocytolysis (grade 2). During acute rejection a decrease in the number of cultures containing predominantly $CD8^+$ cells was observed ($p < 0.025$).

$CD8^+$ $\tau\delta^+$ containing cultures originated from EMB taken more than 1 year posttransplant.

$CD3^-$ cells were found in 21 cultures (19 patients). In these cultures 7% to 96% of cells expressed CD16 and/or CD56 antigens (median 24%) and EMB were taken from 6 to 1587 days posttransplant (median 148 days). In seven cultures from various patients the NK cells were dimly CD8 positive, six of them were grown from EMB taken in the first posttransplant year. We detected significantly more NK cells in cultures from biopsies taken more than 1 year after transplantation compared to the first posttransplant year (8/24 versus 13/176, $p < 0.001$). These cells were never grown from biopsies with myocytolysis.

Cell-mediated cytotoxicity. From 126 EMB (47 patients) sufficient cells were generated to test cytotoxic functions (Table 1). Kill of donor antigen-bearing target cells was found in 106 cultures. Table 4 shows the results of the specific CML reactivity. Donor-specific cytotoxicity against HLA class I antigens (either alone or in combination with reactivity against class II) was found in 75% of the cases, and against class II antigens (alone or in combination with class I) in 56%. Reactivity against both class I and class II antigens was detected in 48% of the bulk cultures and restricted reactivity (i.e., against either class I or class II antigens only) in 37%. Cultures originating from grades 1 and 2 EMB showed multispecific CML reactivity significantly more often when compared to grade 0 EMB cultures (Table 4, $p < 0.01$), while most negative CMLs were found among grade 0 EMB cultures ($p < 0.005$, compared to grades 1 and 2 EMB cultures).

In time a significant shift was observed from a predominantly multispecific to a more restricted CML pattern when the cultures from EMB taken before and after 3 months were compared (Table 4). This proved to be due to a significant decrease in the number of cultures with HLA class II-directed cytotoxicity derived from grades 1 and 2 EMB ($p < 0.01$). This decrease was not accompanied by a significant change in the phenotypic composition of the bulk cultures as CD4 remained the predominant phenotype at any time after transplantation, regardless of the CML specificity of the cultures. Similar phenotypic compositions were found in cultures with HLA class I-directed cytotoxicity and in cultures without donor-directed CML reactivity.

Kill of the K562 cell line was observed in 26/107 tested bulk cultures. In 22 of these allospecific reactivity was also found. NK reactivity did not show a significant relation with time after transplantation or histological rejection grade. Only 7/26 cultures contained cells with the CD16 and/or CD56 phenotype of which two had 70% and 64% $CD8^{dim+}$ NK cells next to $CD4^{bright+}$ T cells. One of the latter cultures showed donor-specific killing next to kill of K562, the other culture did not show donor-specific lysis, but only killed K562. Five of

TABLE 4 CML reactivity of 126 bulk cultures (47 patients) against panel cells sharing either HLA class I or class II with the donor

	CML specificity	Grade 0, 4		Grades 1 and 2		Total	
		n	(%)	*n*	(%)	*n*	(%)
<90 days		*n*[a] = 18		*n* = 59		*n* = 77	
	Class I only	7	(39)	10	(17)	17	(22)
	Class II only	1	(6)	5	(8)	6	(8)
	Both class I and II	5	(28)	39	(66)	44	(57)
	Negative	5	(28)	5	(8)	10	(13)
>90 days		*n* = 16		*n* = 33		*n* = 49	
	Class I only	4	(25)	14	(42)	18	(37)
	Class II only	2	(13)	3	(9)	5	(10)
	Both class I and II	4	(25)	12	(36)	16	(33)
	Negative	6	(38)	4	(12)	10	(13)
Total		*n* = 34		*n* = 92		*n* = 126	
	Class I only	11	(32)	24	(26)	35	(28)
	Class II only	3	(9)	8	(9)	11	(9)
	Both class I and II	9	(26)	51	(55)	60	(48)
	Negative	11	(32)	9	(10)	20	(16)

After 90 days posttransplantation a significant decrease in the number of cultures with generalized reactivity was found. This proved to be due to a decrease in the number of cultures with HLA class II-directed cytotoxicity derived from grades 1 and 2 EMB (from 44/59 to 15/33 after 90 days, $p < 0.01$). Most negative CMLs were found among grade 0 EMB cultures ($p < 0.005$ compared to grades 1 and 2 EMB cultures).

[a] *n* = numbers of cultures.

eight tested CD8⁻ NK cell containing cultures showed NK reactivity, two did not lyse donor antigen-bearing panel cells. All four tested cultures with high percentages of NK cells (>55%) killed K562.

Rejectors versus nonrejectors. The group of 87 heart transplant recipients consisted of 59 patients who had experienced one or more rejection episodes (rejectors) and 28 patients who had never rejected their grafts (nonrejectors). There was no significant difference in the number of HLA mismatches between donor and recipient in both populations (mean number of mismatches 2.78 versus 2.79 for HLA A, B and 1.41 versus 1.21 for HLA DR, respectively).

Generation of lymphocyte cultures from EMB from both patient groups showed similar growth patterns in relation to time after transplantation and histological rejection grade (Table 5). Cultures from biopsies from nonrejectors more often showed a predominance of CD8⁺ T cells than EMB cultures from rejectors (Table 6, $p < 0.025$). This was due to the significantly higher proportion of CD8-dominated cultures derived from histology grade 1 EMB in the nonrejectors ($p < 0.005$ compared with grade 1 EMB cultures from rejectors), especially in grade 1 EMB preceding a grade 0 biopsy. No significant relation with time after transplantation could be demonstrated.

CML panel reactivity of EMB cultures from rejectors changed from a predominantly multispecific pattern in the first 3 months to a more restricted cytotoxicity

TABLE 5 Culture results of EMB from rejectors and nonrejectors in relation to time after transplantation

Days post transplant	Rejectors				Nonrejectors			
	No. patients	No. EMB	Growing *n*	(%)	No. patients	No. EMB	Growing *n*	(%)
0–10	17	17	5	(29)	13	14	2	(14)
11–30	18	44	25	(57)	13	36	21	(58)
31–90	20	72	53	(74)	12	53	38	(72)
91–180	24	73	42	(58)	10	23	13	(57)
181–365	23	59	25	(42)	9	24	6	(25)
>365	36	87	41	(47)	14	33	12	(36)
Total	59	352	191	(54)	28	183	92	(50)

TABLE 6 Predominant phenotype of EMB cultures from 59 rejectors and 28 nonrejectors and relationship with histological rejection grade

	Histological rejection grade	No. cultures				
		>60% CD4	CD4 = CD8	>60% CD8	>60% CD4⁻CD8⁻	Total
Rejectors	0	13	3	13	3	32
	1	39	20	16	2	77
	2	16	8	2	1	27
Total		68	31	31	6	136
Nonrejectors	0	14	1	4	0	19
	1	17	6	22	0	45
Total		31	7	26	0	64

thereafter (Table 7). In the first 3 months 67% of cultures were cytotoxic against both HLA class I and class II, which decreased to 36% thereafter ($p < 0.01$). This was mainly due to a decrease of HLA class II-directed cytotoxicity. In the nonrejectors no significant decline in the fraction of cultures showing multispecific cytotoxicity was found. In the first 3 postoperative months the number of cultures exhibiting cytotoxicity against both HLA class I and II was lower than in the rejectors. After 3 months no significant difference in cytotoxic specificities between rejectors and nonrejectors could be demonstrated.

DISCUSSION

The present study shows that donor-specific cytotoxic lymphocytes can be grown from EMB taken at any time after transplantation, especially from grades 1 and 2 EMB. We also showed that CML panel reactivity changed in relation to time from a predominantly multispecific pattern to a more restricted one, which proved to be due to a significant decrease in the number of cultures with HLA class II-directed cytotoxicity. No corresponding change was observed in the phenotypic composition as CD4 remained the predominant phenotype in most cultures at any time after transplantation. This could mean that only a small fraction of the lymphocytes in the bulk cultures is responsible for the CML specificity, or that the responsible cytolytic T lymphocytes (CTL) have high affinity antigen receptors that do not require CD4 or CD8 molecules to stabilize antigen binding [7]. MacDonald et al. [8] showed that resistance of murine CTL clones to inhibition with anti-CD8 monoclonal antibodies correlates with in vivo priming

TABLE 7 CML specificity of 87 bulk cultures from rejectors (33 patients) and 39 cultures from nonrejectors (14 patients)

	CML specificity	Rejectors		Nonrejectors	
		n	(%)	*n*	(%)
<90 days		*n*[a] = 48 (18 patients)		*n* = 29 (12 patients)	
	Class I only	7	(15)	10	(34)
	Class II only	2	(4)	4	(14)
	Both class I and II	32	(67)	12	(41)
	Negative	7	(15)	3	(10)
>90 days		*n* = 39 (23 patients)		*n* = 10 (6 patients)	
	Class I only	14	(36)	4	(40)
	Class II only	5	(13)	0	(0)
	Both class I and II	14	(36)	2	(20)
	Negative	6	(15)	4	(40)

In the rejectors a significant decline in the number of cultures with generalized reactivity was found (32/48 versus 14/36, $p < 0.01$). This was mainly due to a decrease of HLA class II-directed cytotoxicity (34/48 at <90 days versus 19/39 at >90 days). In the nonrejectors this was less apparent.

[a] *n* = numbers of cultures.

of these cells. Whether T cells carrying high affinity TCR preferentially home in the allograft has not been investigated yet in the human.

Another finding in the present study was the higher incidence of NK cells and TCR γ/δ^+ cells among cultured graft infiltrating cells at 1 year after transplantation which, with one exception for the $\gamma\delta^+$ cells, only originated from EMB without myocytolysis. The role of these cells in an allograft is not clear. They might play a role in maintaining graft stability or just be attracted to the site by lymphokines produced by major histocompatibility complex (MHC)-restricted cells, macrophages, or other types of cells. Whether $\gamma\delta^+$ cells or NK cells play a role in the development of chronic rejection is still under investigation.

Part of the cultures dimly expressed CD8. These CD8 molecules did not seem to function as an accessory molecule mediating class I-directed killing, for none of the bulk cultures containing high percentages of these cells showed donor-specific lysis. Some of the $\gamma\delta^+$ cell-containing cultures were cloned. Neither donor-specific cytotoxicity nor proliferation could be demonstrated in any of the $CD8^{dim+}$ or the $CD8^-$ $\gamma\delta^+$ clones [9]. Furthermore, after culture of $CD8^+$ clones from one biopsy in recombinant IL-2-containing medium the CD8 expression disappeared (data not shown). The CD8 molecule might have been induced on the cells by the culture conditions in medium with a mixture of lymphokines, including IL-4. It has been demonstrated before that $CD8^{bright}$ can be induced on $CD4^+$ lymphocytes under the influence of IL-4, and that this molecule can be functional in class I-mediated killing [10].

Several comparative studies of the CD4/CD8 profile of the T cells seen in situ (immunoperoxidase staining) and that of the lymphocyte cultures showed that the CD4/CD8 composition of lymphoid cells cultured from biopsies had a good correlation with the actual situation in the graft [11, 12]. The present study shows that most biopsies yield mixtures of $CD4^+$ and $CD8^+$ cells with CD4 as the predominant phenotype in most cases, which is in agreement with data of Fung et al. [13]. In a limited number of observations others found CD8 as the predominant T-cell subset in cultures from renal or cardiac biopsies. This is thought to be due to the use of azathioprine in their immunosuppressive protocols [2, 12, 14]. Like the present study, no relation between predominant phenotype and the number of HLA mismatches between donor and recipient was found.

We showed that a lower rejection grade was associated with a higher number of CD8-dominated cultures. Also in patients who never experienced acute rejections, significantly more $CD8^+$ cells were found among infiltrating cells. These observations could suggest that these cells play a mitigating role in the rejection process. An alternative explanation for the greater predomination of $CD4^+$ cells during rejection might be that it is a consequence of higher HLA class II expression on graft tissue. We could not confirm the finding of Weber et al. [15], that cell growth from histologically negative EMB obtained in the first postoperative month had a positive correlation with the cumulative incidence of subsequent histological rejection.

The lower incidence of HLA class II-directed cytotoxicity after the first 3 months in the present study is in agreement with an extensive survey among renal transplant recipients [16]. This survey showed that the effect of DR matching on the relative risk for graft failure was high in the first 5 months, thereafter the effect disappeared. On the other hand, the matching effect of HLA class I antigens was evident during the whole follow-up period. In the present study we showed that class I-directed cytotoxicity remained relatively constant in time after transplantation. Several investigators have demonstrated the induction of MHC class I antigens on myocytes and increased expression of class I and II on interstitial structures during rejection [17–19], which makes the graft tissue more vulnerable to specific cell-mediated lysis [20]. In some studies MHC expression in the allograft returned to normal after successful rejection treatment [18], although others found persistence of expression of donor-type class II determinants on interstitial structures of the donor heart 1 and 2 years after transplantation [17, 21]. It has been shown that, to a certain degree, HLA class II expressing dendritic cells of donor origin are replaced by recipient bone marrow derived cells [17, 22, 23]. This may contribute to the lower incidence of donor-class II directed cytotoxicity. In vitro experiments have shown that lymphokines, particularly interferon γ, regulate the induction and upregulation of (donor) MHC expression on graft tissue and leukocyte binding and penetration through the endothelium [24, 25], and that in the early posttransplant period, when the incidence of acute rejection is high, lymphokine-producing cells are numerous in the graft [26]. This is in agreement with our finding that the highest growth rates of alloactivated lymphocytes were found in the second and third months after transplantation. This peak of growth was observed in the rejector as well as nonrejector patient groups. Apparently the presence of activated lymphocytes in the graft does not always lead to allograft destruction. We found that cultures from nonrejector EMB taken in the first 3 postoperative months generally show a more restricted cytotoxicity than those from rejectors, which indicates that in this period the number of alloreactive CTL clones in vivo is lower in nonrejectors. This might have consequences for the development of myocyte injury.

Many speculations have been made about possible

mechanisms involved in stabilization of the graft and on the role different kinds of cells and lymphokines play in this process [27–30]. Mechanisms that have been proposed to be involved are clonal deletion of antidonor alloreactive cells or specific suppression of alloreactive T cells. From earlier studies on circulating mononuclear cells CML hyporesponsiveness [27, 28] and a reduction in the frequency of donor-reactive CTL precursors [29] have been reported in patients with well-functioning grafts. In contrast, another study on a patient with a well-functioning kidney graft showed that the frequency of donor-specific CTL precursors was still high, but these cells were not operational in vivo [30]. We showed that biopsy-grown lymphocytes from allografts without acute rejection often still contain donor-directed cytotoxic cells. The mechanism that plays a major role in controlling the immune response in vivo is still unclear. Further investigations will address the question of whether specificity of graft infiltrating cells becomes more restricted because of diminished MHC class I and/or class II expression on donor heart tissue, or if it is caused by deletion or suppression of certain allospecific CTL clones, irrespective of the degree of allograft-MHC expression.

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