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EFFECTOR MECHANISMS IN GRAFT-VERSUS-HOST DISEASE IN RESPONSE TO MINOR HISTOCOMPATIBILITY ANTIGENS

I. ABSENCE OF CORRELATION WITH CYTOTOXIC EFFECTOR CELLS[1]

CÉCILE A. C. M. VAN ELS,[1,3] ASTRID BAKKER,[2] AEILKO H. ZWINDERMAN,[4] FERRY E. ZWAAN,[5] JON J. VAN ROOD,[2,5] AND ELS GOULMY[2]

Department of Immunohaematology and Blood Bank, University Hospital Leiden, 2300 RC Leiden, The Netherlands

Cell-mediated immunity against minor histocompatibility (mH) antigens is assumed to contribute to the development of graft-vs.-host disease in recipients of HLA-identical bone marrow grafts. To investigate the role of antihost-specific cytotoxic effector cells, we analyzed patients' T cell cultures after transplantation, in a chromium release assay using T lymphoblasts from patients and donors as target cells. Sixteen patients were studied between 1 and 25 months after grafting. Specific antihost cytotoxic T cell activity was detected in 4 of 5 patients with acute GVHD and in 5 of 5 patients with chronic GVHD, but also in 5 of 6 patients without any clinical signs of GVHD. Generally, the antihost Tc cell activity appeared within the first 3 months, increased to a maximum between 3 and 6 months, and gradually disappeared thereafter. This time effect was significant ($P = 0.002$). There was a suggestive trend in patients with chronic GVHD toward developing higher and more persistent levels of antihost Tc cell activity than in those without GVHD. Yet, these results can no longer support our earlier finding that the generation of antihost Tc cells is associated with the development of GVHD, since antihost Tc cells could be generally detected whether or not GVHD occurred. Our findings do not a priori exclude an effector cell role for Tc cells in GVHD but strongly indicate that other risk factors must be involved as well.

Allogeneic bone marrow transplantation is increasingly used to cure a variety of hematological disorders, such as aplastic anemia and leukemia (*1*). However, transplantation reactions such as graft-vs.-host disease and graft rejection are two major limits to the success of this treatment (*2–4*). In HLA-identical transplantation, the degree of mismatching for so-called minor histocompatibility (mH)* antigens between donor and host is thought to influence the likelihood of GVHD and graft rejection (*5*). Unfortunately, these T cell–mediated host-graft interactions seem to be related in a mutually exclusive way (*6*), such that attempts to prevent GVHD likely increase the incidence of graft rejection and vice versa (*7, 8*). A better understanding of the cellular mechanisms underlying GVHD and graft rejection is presently needed to manage this dilemma.

The involvement of antihost-specific cytotoxic T cells as effector cells in GVHD induced to mH antigens was first suggested by experimental studies in mice. Anti–recipient strain Tc cells could be demonstrated in spleens of mice undergoing GVHD (*9*). Likewise, antihost cytotoxic effector cell populations could be isolated from patients achieving GVHD after HLA-identical BMT (*10, 11*). Recently, we showed that the presence of mH antigen–specific Tc cells shortly after grafting was associated with the development of chronic GVHD (*5, 12*). Further, it was demonstrated by Tsoi et al. (*13, 14*) that cytotoxicity of patients' post-BMT lymphocytes against host fibroblasts in a cellular inhibition assay tended to correlate with the occurrence of acute GVHD. Interestingly, these latter studies suggested that the time of onset of GVHD was crucial for the detection of the antihost cytotoxicity, which emerged only between one and two months after the development of GVHD. To gain insight into this time effect and to extend our earlier observations, we here investigated the antihost-specific Tc cell reactivities in sixteen patients sequentially after transplantation. The results are consistent with our previous studies in that strong, host-directed Tc cells emerge in all patients with chronic GVHD, but differ with regard to the incidence in patients without GVHD and with acute GVHD. Our findings may have a bearing on current concepts of the biological role of antihost-specific Tc cells in BMT.

MATERIALS AND METHODS

Patients. Sixteen patients (6 male, 10 female) received bone marrow from their HLA-A, -B, -Cw, -DR–identical, MLC-nonreactive sibling donors. As a conditioning regimen for transplantation, 15 patients with leukemia received cyclophosphamide (60 mg/kg/day ×2) and total-body irradiation (8 Gy), 1 patient with aplastic anemia received cyclophosphamide (50 mg/kg/day ×4) and total-lymphoid irradiation (2 Gy/day ×10). To prevent acute GVHD, 11 patients were given methotrexate and 5 patients were given cyclosporine A. All patients lived at least 100 days after grafting and thus were at risk for acute and chronic GVHD. Acute GVHD was diagnosed in 10 patients, 5 of whom later developed chronic GVHD. Clinical information on the patients is summarized in Table 1. The standard protocol for MTX was 15 mg/m^2 i.v. on day 1, 10 mg/m^2 on days 3, 6, 9, and 11 after transplantation; and thereafter weekly for the first 100 days. CsA was given as a continuous i.v. infusion, 3 mg/kg/day, started on day −1, followed by oral administration (9 mg/kg/day). The dose was adjusted according to the clinical course, renal function, and plasma concentration of CsA

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[2] Department of Immunohaematology and Blood Bank, University Hospital Leiden.

[3] Address correspondence to: C A C.M. van Els, Department of Immunohaematology and Blood Bank, University Hospital Leiden, P.O. Box 9600, 2300 RC Leiden, the Netherlands.

[4] Department of Medical Statistics, University of Leiden.

[5] Department of Haematology and Bone Marrow Transplantation, University Hospital Leiden.

* Abbreviations: mH, minor histocompatibility; PLT, primed lymphocyte test,

TABLE 1. Clinical information concerning the patients

	Patients						Living or cause of death	Graft-versus-host disease				Period of in vitro testing
	upc[a]	Age	Sex	Sex mismatch	Diagnosis	Survival after grafting		Prophylaxis	Acute GVHD		Chronic GVHD	
1	6500	33	M[b]	No	ANL[c] 1°[d]	>760[e]	Alive	MTX[f]	0[g]	—	0[h]	40–187[e]
2	6545	31	M	Yes	ALL 2°	538	Leukemia	MTX	0	—	0	36–336
3	6223	42	F	Yes	ANL 1°	>760	Alive	MTX	0	—	0	43–677
4	6217	20	F	Yes	ANL 1°	609	Leukemia	MTX	0	—	0	139–382
5	6346	24	M	Yes	ANL 1°	>760	Alive	MTX	0	—	0	43–711
6	6825	17	F	No	ALL 1°	>760	Alive	MTX	0	—	0	124–697
7	6284	21	M	Yes	SAA	143	Transplant	MTX	II	34[i]	0	40–97
8	7035	36	F	Yes	ANL 1°	>760	Alive	MTX	II	54	0	97–773
9	7431	38	F	No	ANL 1°	>760	Alive	CsA	I	12	0	29–729
10	6067	15	M	No	ANL 1°	>760	Alive	MTX	II	33	0	50–773
11	7288	38	F	Yes	ANL 1°	175	Transplant	CsA	II	14	0	29–158
12	6072	26	F	Yes	ALL 2°	>760	Alive	MTX	II	18	1	25–773
13	6966	32	F	Yes	ANL 1°	394	Transplant	MTX	II	28	2	33–382
14	7448	38	F	No	ANL 1°	267	Transplant	CsA	III–IV	12	2	27–180
15	7037	40	M	No	ANL 1°	137	Transplant	CsA	III–IV	11	2	22–137
16	7384	39	F	No	ANL 1°	>760	Alive	CsA	I	17	1	29–572

[a] Unique patient code.
[b] M: male; F: female.
[c] ANL: acute nonlymphoblastic leukemia; ALL: acute lymphoblastic leukemia; SAA: severe aplastic leukemia.
[d] 1°, 2°: first, second remission.
[e] Days after transplantation.
[f] MTX: methotrexate; CsA: cyclosporine.
[g] Acute GvHD grade.
[h] Chronic GVHD grade: 0 = none; 1 = limited; 2 = extensive.
[i] Day of onset of acute GVHD.

(600–1000 μg/ml). Generally, the oral dose was slowly tapered over a period of months with the aim of discontinuing it at 3–4 months after transplantation. As therapy for GVHD, patients received prednisone (1–2 mg/kg/day for 10–14 days, then tapered) or high-dose methylprednisolone (20 mg/kg/12 hr for 2 days, then tapered). Patients who developed chronic GVHD were treated with prednisone (1 mg/kg/day) and/or azathioprine (2 mg/kg/day).

Blood samples. Heparinized blood samples were collected from recipients before and periodically after transplantation, from the sibling bone marrow donors, and from HLA-typed unrelated individuals. The patients were scheduled to be tested at days 30, 60, 90, 180, 360, and 720—missing intervals were caused by early lymphopenia or by the death of patients (see Table 1). Peripheral blood leukocytes were isolated by ficoll-isopaque density gradient centrifugation, washed, and resuspended in RPMI 1640–dimethylsulfoxide (final concentration 10%) for cryopreservation in liquid nitrogen.

Tissue culture medium. All T cell cultures were performed in RPMI 1640 supplemented with antibiotics (gentamycin) and 15% human serum.

Generation of host- and allo-HLA-specific T cell lines. T cell lines in the GVH direction were generated as follows; 4×10^6 responder cells, either PBL from patients (n = 16) obtained sequentially after transplantation or PBL from bone marrow donors (n = 12), as unprimed controls were stimulated with 4×10^6 20 Gy irradiated patients' pretransplantation PBL as host-specific antigen-presenting cells. At day 6, responder T cells were specifically restimulated and further propagated as described elsewhere (*15*). To study HLA-specific T cell alloreactivities, the patients' blood samples up to 6 (or 9, see the accompanying article) months after BMT were, in a separate analysis, also sensitized against irradiated stimulator cells from an unrelated HLA-mismatched donor. To limit the experimental variation, for each patient the long term antihost and anti-HLA –reactive analyses (Table 2) were carried out simultaneously.

Phenotype analysis. Patients post-BMT T cell lines were stained for CD3, CD4, and CD8 expression by a standard double immunofluorescence technique, using PE- and FITC-conjugated Leu4 (CD3), Leu3a (CD4), and Leu2a (CD8) monoclonal antibodies, and were assayed on a fluorescence activated cell sorter. All samples tested (n = 52) were $CD3^+$ (94±8%) and showed an equal mean distribution of $CD4^+$ cells (51±27%) and $CD8^+$ cells (46±28%, data not shown).

TABLE 2. Flow chart of the sensitization and the functional analysis of host antigen and HLA antigen specific T cell lines

In vitro sensitization phase			Effector phase CML[a]/PLT[b] (target[d]/stimulator[e])
Alloantigen	Responder	Stimulator[c]	
mH	Recipient[f]	Host[g]	Host/donor
	Donor	Host	Host/donor
HLA	Recipient	Unrelated	Unrelated/donor
	Donor	Unrelated	Unrelated/donor

[a] Functional assay in this study.
[b] Functional assay in the accompanying article (*7*).
[c] On days 0 and 6 (PBL).
[d] PHA-stimulated lymphoblasts.
[e] PBL.
[f] At different times after BMT.
[g] Derived from recipients before transplantation.

Cell-mediated lympholysis asssay. Tc cell activity was determined using 3–4-weeks-old T cell lines from patients and donors as effector cells in a standard ^{51}Cr release assay (*16*). Target cells were T lymphoblasts derived from the original stimulator—i.e., from the host before transplantation to measure the host-specific reactivities, or from the HLA-mismatched unrelated donor to measure the HLA antigen-directed reactivities. T lymphoblasts from the bone marrow donor were used as an autologous control. Briefly, 5×10^3 ^{51}Cr-labeled T lymphoblasts, generated by treatment of PBL with 1% PHA mitogen (Difco) for 3 days and expanded on Il-2, were incubated at 37°C for 4 hr together with 2×10^5 effector cells (E:T = 40:1). Then the supernatants were harvested for counting in a gamma counter (Packard Instru-

ments). Percentages of specific ^{51}Cr release were calculated according to the following formula: ER-SR/MR-SR × 100%, in which experimental, spontaneous, and maximal release (ER, SR, and MR) were, respectively, the ^{51}Cr release by target cells measured in the presence of effector cells (ER), in culture medium alone (SR), and in culture medium with the detergent zaponine (MR).

Statistical methods. A multivariate analysis of variance model (Manova) (*17*) was applied, containing group-, time-, and group-by-time interacting terms, to determine the levels of significance of the differences in antihost Tc cell activity between different intervals after transplantation and between different patient groups achieving no, acute, or acute followed by chronic GVHD.

RESULTS

Evidence that posttransplantation Tc cell activities are specifically directed against the host. T cell lines were induced from the PBL of 16 patients, obtained at different times between 1 and 25 months after HLA-identical BMT, which were sensitized with patients' own pretransplantation PBL. Thirteen patients were studied 3 or more times after grafting, whereas 3 patients were only studied twice. We analyzed a total of 60 T cell cultures for the presence of Tc cell reactivities against host and donor target cells in a ^{51}Cr release assays (Table 2). As is shown in Table 3, significant cytotoxicity (i.e., more than 20% of lysis) against host target cells was detected in 39 of the 60 host-sensitized cultures, whereas no cultures showed Tc cell activity against the donor. Thus, all activities detected were host-specific.

Absence of in vitro generation of antihost-specific Tc cell activities from unprimed donors. To investigate whether the host-specific cytotoxicity could possibly have been introduced by in vitro manipulation, cytotoxic assays were also performed with T cell lines derived from 12 marrow donors (Table 2). The Tc cell activities against host as well as donor targets were negligible in 11 of 12 donor T cell lines (Table 3). One female donor, who was sensitized in vivo by pregnancies, showed Tc cell reactivity specifically against host target cells. Hence, antihost Tc cell reactivity could not usually be generated in vitro.

Kinetics of the antihost specific Tc cell activities after transplantation. The 39 cell cultures with antihost-specific Tc cell activity (Table 3) originated from 14 of 16 patients (Fig. 1). Two patients (2 and 8) remained negative for at least one year and two years, respectively. The other 14 patients showed considerable variation with respect to the magnitude and the kinetics of their antihost Tc cell activities. In some patients more than 20% of antihost lysis was found in all samples (1,7,11,12,13, and 16) while in other patients cytotoxicity was only demonstrated in some samples (3,4,5, 6,9,10,14, and 15). Of the 14 patients who achieved antihost Tc cells, 12 were evaluated during the first 3 months (1,3,5,7,9–16). In 10 of these 12 patients the Tc cell activity already appeared within this period (1,3,6,7,10,11,12,13,14, and 16). Thereafter, the activity tended to increase to a maximum level between 3 and 6 months. Variance analysis showed that the average antihost Tc cell activity in the latter interval (61±7%) was significantly higher than the averages found between 0 and 1.5 months (31±6%, $P = 0.004$) and between 1.5 and 3 months (39±7%, $P = 0.039$). Although Tc cell activities persisting for at least two years could be found in a few patients (e.g. 12 and 16), Tc cell activities gradually decreased after 3–6 months (e.g., in patients 3,4,5,9, 10, and 13). This trend was also significant. The average antihost Tc cell activity between 9 and 12 months (49±7%) was significantly lower than between 3 and 6 months (61±7%, $P = 0.005$), while the average antihost Tc cell activity between 12 and 25 months (24±7%) was significantly lower than between 3 and 6 months ($P = 0.001$) and 6 and 9 months (49±7%, $P = 0.012$).

Early regeneration of Tc cell activity against HLA alloantigens. To test the possibility that the failure to detect antihost Tc cells shortly after grafting as observed in several patients might reflect a general Tc cell impairment (as a result of incomplete T cell reconstitution or immunosuppression) rather than a specific nonresponsiveness to host antigens, we analyzed the capacity of the 16 patients' lymphocytes up to 6 months to mount an in vitro Tc cell response to HLA alloantigens (Table 2). All lymphocyte samples that were sensitized against HLA alloantigens (n = 29) showed strong Tc cell activity against the original stimulator cells (84±20% lysis) but not against the autologous donor cells (1±3% lysis, data not shown). Thus, the Tc cell responses against HLA alloantigens were functionally restored in all patient samples studied.

Absence of correlation between antihost-specific Tc cell activities and the development of acute or chronic GVHD. The antihost Tc cell activities observed in patients without GVHD (Fig. 1A) were compared with the results obtained in patients who developed acute GVHD (Fig. 1B) or acute and subsequently chronic GVHD (Fig. 1C). Variance analysis showed no statistically significant differences between groups. All patients who achieved chronic GVHD (n = 5) generated antihost Tc cells; in 3 of these (12, 13, and 16) extremely high cytotoxicity levels were observed shortly after grafting, while 4 of 5 patients with acute GVHD, and 5 of 6 patients without GVHD also developed Tc cells. The statistical analysis showed that the average antihost Tc cell activity in patients with chronic GVHD (51±9%) was higher than in patients without GVHD (28±9%) or with acute GVHD (38±10%)—however, these differences were not significant.

TABLE 3. Detection of host-specific Tc cell activity in patients' post-BMT and donors' T cell lines

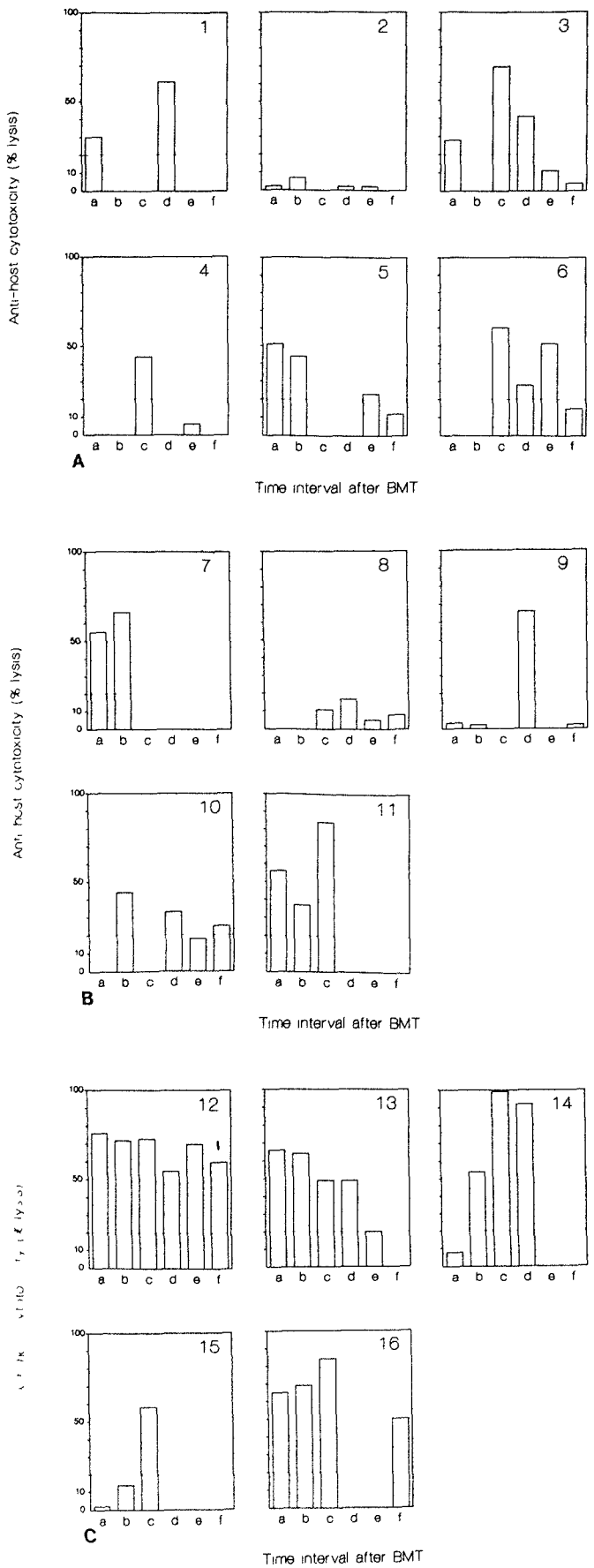
		Anti-donor cytotoxicity	
		Yes	No
Anti-host cytotoxicity	Yes	0/60[a]	39/60
		0/12[b]	1/12
	No	0/60	21/60
		0/12	11/12

[a] Patients' post-BMT T cell lines ($n^{total} = 60$).
[b] Donor-derived T cell lines ($n^{total} = 12$).

DISCUSSION

In our earlier studies we found an association between the presence of circulating antihost-specific Tc cells at about 40 days after BMT and the development of chronic GVHD (*5, 12*). Currently, with longer-term studies for antihost Tc cells, we observed that patients could be unresponsive until or even beyond day 40, but still could develop an antihost Tc cell response in a later phase. However, the main finding of this study, which does not support our previous conclusion, is that antihost Tc cells seem to occur frequently, and not only in patients with chronic GVHD.

The presence of host-specific Tc cells in 5 of 6 patients without GVHD clearly demonstrated that an antihost immune



response can emerge after transplantation whether GVHD develops clinically or not. Likewise, in the mouse, splenic Tc cells with host reactivity have been detected in recipients without any sign of GVHD after mH antigen–disparate BMT (*18*). Clearly, this absence of correlation poses questions as to the role of classic cytotoxic cells in the process of GVHD. It might be that the relevance of antihost-specific Tc cells relates to the tissue distribution of their target antigens. As yet, little is known about the expression of mH antigens. Recently we found that several Tc cell–defined mH antigens were differentially expressed on human bone marrow precursor cells and in the skin (*19* and De Bueger MM, et al., manuscript in preparation). Hence, these observations suggest that some, but not all, mH antigens might play a role as target antigens in graft rejection and GVHD.

Another possibility is that, while antihost Tc cells could become cytotoxic when stimulated in vitro, they may be held in an "anergic" state in vivo. In this respect our observations in patients without GVHD may relate to the discordances described between in vivo and in vitro assays in recent studies of immunologic tolerance in man as well as in the mouse (*20–22*). Although the mechanism of in vivo anergy remains unclear, the administration of immunosuppressive agents to patients might play a role. Here it is noteworthy that, while in vitro cytotoxicity was observed in nearly all cases, patients receiving CsA (n = 5) all developed GVHD—whereas, of those receiving MTX, only 5 of 11 developed GVHD. Whether these findings have a bearing on the capacity of MTX versus CsA to establish in vivo tolerance, however, cannot be concluded from our studies. Evidence that graft-host tolerance after human BMT might be mediated by suppressor cell activity was provided by Tsoi et al. (*23*), who showed that the lymphocytes from long-term survivors without GVHD could specifically suppress an in vitro model of the GVHD reaction, but that lymphocytes from survivors with chronic GVHD could not.

Finally, it should be stressed that the issue of whether GVHD-related tissue damage is brought about by classic Tc cells is still unsettled. Therefore, it is of importance to investigate other possible risk factors, such as antihost T helper cells (*24*), autocytotoxic null cells (*25*), and large granular lymphocytes (*26*), as well as the effects mediated by cytokines (*27*). The possible involvement of antihost Th cells is emphasized in another study, wherein we show a significant correlation between these cells and the development of acute GVHD (see the accompanying article).

Analysis of the relation between the time of onset of GVHD and the initiation of in vitro antihost Tc cell activity would provide further insight into the role of Tc cells. Unfortunately, blood samples preceeding the diagnosis of GVHD were not available (Table 1). In some cases we observed a clear lag

FIGURE 1. Kinetics of host-specific Tc cell activity: (A) patients without GVHD, (B) patients with acute GVHD, (C) patients with chronic GVHD. Bars represent sequential Tc cell activity (% lysis) against host lymphoblasts of posttransplantation T cell lines (E:T = 40:1) derived from patients 1 to 6 without GVHD (A), from patients 7 to 11 with acute GVHD (B), and from patients 12 to 16 with acute and subsequently chronic GVHD (C). Intervals after BMT in months are: a: 0–1.5; b: 1.5–3; c: 3–6; d: 6–9; e: 9–12; f: 12–25 The absence of bars means that no material was available from the interval. No Tc cell activity against autologous donor T cell blasts was detected in any of the T cell cultures (Table 3).

between the onset of GVHD and antihost Tc cell activity, whereas in others we found that antihost Tc cell activity could be present as early as a few days after clinical diagnosis of acute GVHD. In contrast, Tsoi et al. (*14*) found that in vitro antihost cytotoxicity measured against fibroblasts could only be optimally demonstrated between 1 and 2 months after the onset of GVHD. Furthermore, these authors observed a lower incidence of antihost cytotoxicity. Therefore, our method, using specifically in vitro restimulated effector T cell lines and lymphoblasts as target cells, seemed to be more sensitive for the detection of antihost cytotoxicity. Alternatively, the two methods could be directed against different antigens. In this study, the levels of Tc cell activity increased to a maximum between 3 and 6 months after grafting (Fig. 1). Whether this trend reflects an in vivo strengthening of the antihost response or is related to the suppressive regimen remains unknown. The sharp increase in Tc cell activity found in patients 9, 14, and 15, which coincided with the interruption of CsA administration, favors the latter option—yet patients having high antihost Tc cell activity in the course of MTX or CsA were also found (e.g., 12,13, and 16). If the immunosuppression indeed may account for the Tc cell nonresponsiveness observed in some cases, this effect is host-specific, since we found normal cytotoxicity against HLA alloantigens in all patients during prophylaxis.

Our study favors the idea that, more often than expected, interactions between host mH antigens and Tc cell subsets are initiated after HLA-identical BMT. Apparently, the generation of specific antihost Tc cells does not necessarily result in an in vivo antihost attack, so the role of classic cytotoxic effector cells in human GVHD is questioned. Immunogenetic studies with cloned cells specific for single mH antigen specificities and identification of the "major" minors—i.e., those triggering an injurious Tc cell subset—will be necessary to settle this issue.

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