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Goulmy, E.A.J.M.; Els, C.A.C.M. van; Bakker, A.; Zwinderman, A.; Zwaan, F.E.; Rood, J.J. van

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

II. EVIDENCE OF A POSSIBLE INVOLVEMENT OF PROLIFERATIVE T CELLS¹

CÉCILE A.C.M. VAN ELS,^{2,3} ASTRID BAKKER,² AEILKO H. ZWINDERMAN,⁴ FERRY E. ZWAAN,⁵
JON J. VAN ROOD,⁵ AND ELS GOULMY²

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

To study helper T cell activation against minor histocompatibility (mH) antigens of the host after HLA-identical bone marrow transplantation, patients' lymphocytes collected longitudinally after transplantation were tested in a primed lymphocyte test using PBL from patients and donors as stimulator cells. Sixteen patients were studied between 1 and 25 months after grafting. Antihost Th cells were detected in 10 patients. Optimum levels of antihost activity were generally reached within the first 3 months, thereafter two patterns were identified; in some patients the antihost Th cell activity persisted for at least 2 years, whereas in other patients a decline was observed with time.

Antihost Th cell activity developed in each of 5 patients with acute GVHD, in 3 out of 5 patients with chronic GVHD, but in only 2 out of 6 patients without GVHD. The average antihost Th cell activity in patients with acute GVHD was significantly higher than in patients without GVHD ($P=0.036$) and was also higher, although not significantly, than in patients with chronic GVHD.

These findings indicate that, in man, as was shown in studies in mice, helper T cells do participate in the response to mH antigens. Although other mechanisms may also be involved, we here propose that mH antigen-specific Th cells may be a risk factor for acute GVHD.

Treatment of patients with leukemia or aplastic anemia with allogeneic bone marrow transplantation (BMT) from HLA-identical siblings has become widely used in the last decade (1, 2). As a consequence of incompatibility for minor histocompatibility (mH)* antigens, moderate to severe graft-vs.-host disease (GVHD) may develop in 10-50% of the patients (3, 4). Although the role of T cells in the etiology of GVHD is beyond question, the involvement of individual T cell subsets of the cytotoxic and the helper phenotype is much less clear. Experimental studies were carried out in which purified T cell sub-

populations were grafted between mouse strains that were identical at the MHC (H-2), differing only for mH antigens (5, 6). Based on a vast amount of experiments showing that murine GVHD was solely caused by $Lyt2^+$ cells without an apparent role for $L3T4^+$ T cells (6-8), it was for quite some time assumed that classic cytotoxic cells were the principle effector cells mediating murine GVHD to mH antigens. However, further studies showed that this phenotypic correlation was not an invariable finding (9), and that in certain mH antigen-incompatible strain combinations $L3T4^+$ cells also could induce or worsen the development of GVHD (10, 11).

In man, much work on the effector cell mechanisms of GVHD in HLA-identical grafting has been concentrated on the role of cytotoxic effector T cells. Host-directed Tc cells were successfully isolated from bone marrow recipients (12-14) and seemed to correlate with the development of GVHD (4, 14). In the accompanying article concerning the longitudinal behavior of antihost Tc cells, however, we show that, in more cases than had been expected, these Tc cells emerged, regardless of whether GVHD developed. From that study we concluded that other risk factors probably are involved also in the graft-vs.-host attack. The present study was undertaken to evaluate in the same patient material the role of one such alternative—a delayed-type hypersensitivity-like response. Host-directed proliferative T cells have rarely been described in patients having GVHD (15, 16). Here we report on the long-term kinetics of T helper cells in response to host mH antigens in 16 patients. Analogous to what was observed in the mouse system, our findings point to a possible involvement of antihost Th cells in the development of GVHD in man.

MATERIALS AND METHODS

Patients, bloodsamples, tissue culture medium, generation of host- and allo- HLA-specific T cell lines, and phenotype analysis. These have been described elsewhere. (See the accompanying article).

Proliferation assay Proliferative T cell activity was determined using

TABLE 1. Detection of host-specific Th cell activity in patients' post-BMT and donors' T cell lines

Antihost proliferation	Antidonor proliferation	
	Yes	No
Yes	4/57 ^a	21/57
	3/12 ^b	1/12
No	0/57	32/57
	0/12	8/12

^a Patients' post-BMT T cell lines ($n^{\text{total}}=57$).

^b Donor-derived T cell lines ($n^{\text{total}}=12$).

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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.

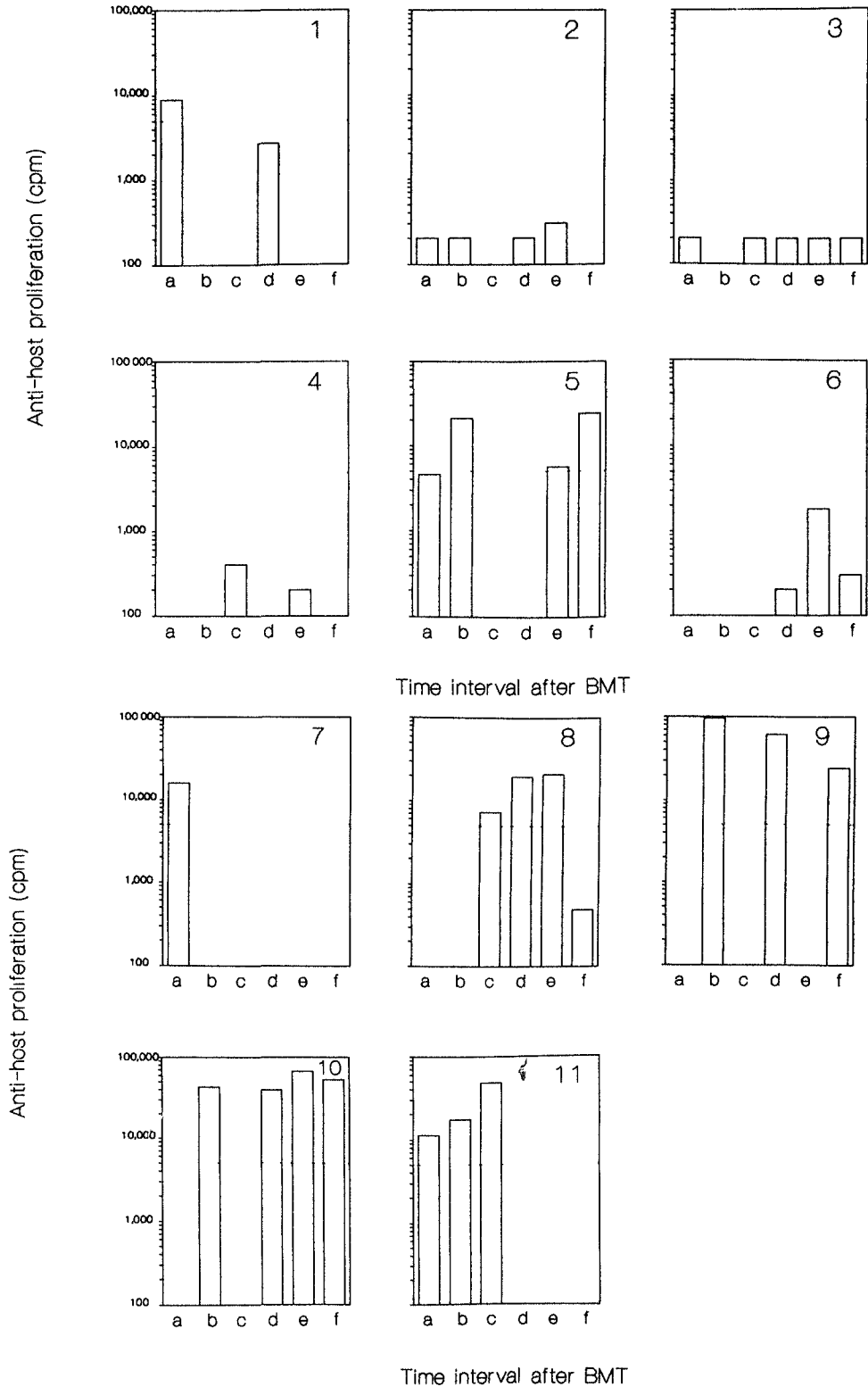


FIGURE 1. Kinetics of host-specific Th cell activity: (A) patients without GVHD; (B) patients with acute GVHD; (C) patients with chronic GVHD. Bars represent sequential antihost Th cell activity (in cpm) of posttransplantation T cell lines derived from patients 1 to 6 without GVHD (A), from patients 7 to 11 with acute GVHD (B), and from patients 12 to 16 with acute and subsequently chronic GVHD (C). Host PBL were used as stimulator cells in PLT. Intervals after

transplantation (in months) are (a) 0-1.5; (b) 1.5-3; (c) 3-6; (d) 6-9; (e) 9-12; and (f) 12-25. The absence of bars means no material was available from the indicated interval. No cultures showed Th cell activity against autologous donor PBL except two cultures from patient 10 (1.5-3 months, 17,100 cpm; 6-9 months, 18,900 cpm), one culture from patient 8 (9-12 months, 41,100 cpm), and one culture from patient 11 (1.5-3 months, 7800 cpm).

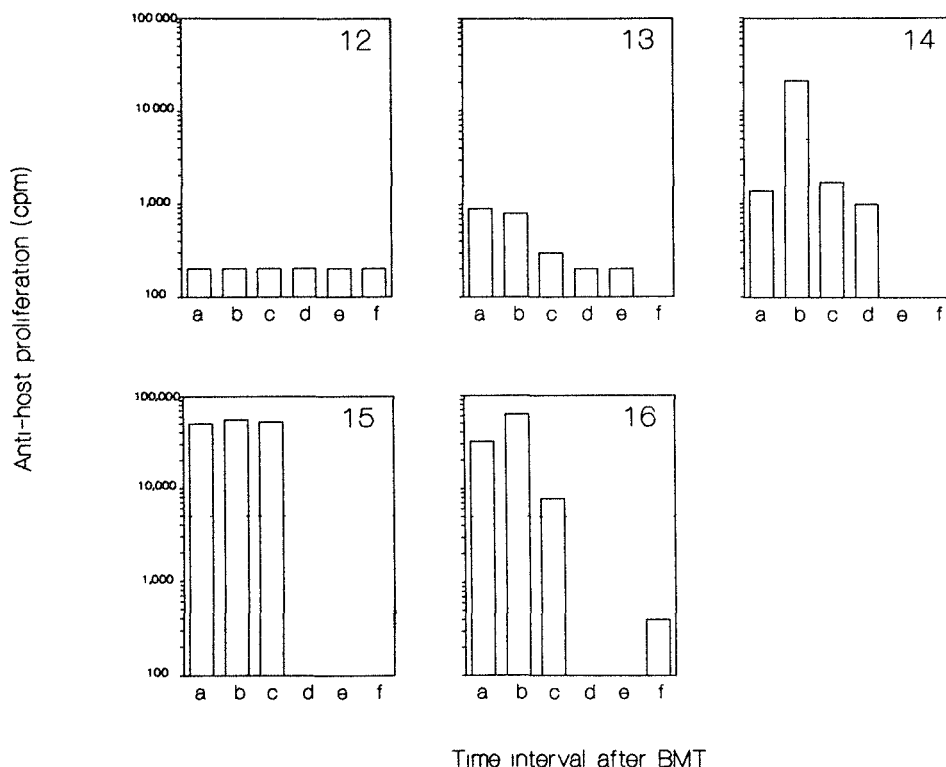


FIGURE 1 con't

3-4-week-old T cell lines from patients and donors as responder cells in a standard primed lymphocyte test (PLT) assay (17) in the presence of the original stimulator cells—i.e., PBL from patients before transplantation to measure host-specific reactivities and from the HLA mismatched unrelated donors to measure HLA alloantigen-specific reactivities. PBL from the bone marrow donors were used as an autologous control. T cells (1×10^6) were cultured with 1×10^6 20 Gy-irradiated PBL in flat-bottomed microtiter plates for 64 hr. At 16 hr before harvesting, the cultures were labeled with $1 \mu\text{Ci}$ tritiated thymidine. Isotope incorporation was measured in a liquid scintillation counter. The results were expressed as the mean counts per minute of triplicate cultures. Standard deviations were below 20% or medium background.

Statistical methods A multivariate analysis of the variance (MANOVA, see the accompanying article) was applied to determine the statistical significance of differences between Th cell activities found in different intervals or groups of patients. The data were computed after logarithmic transformation.

RESULTS

Host specificity of the posttransplantation Th cell activities T cell lines were induced from PBL of 16 patients sequentially after HLA-identical BMT by sensitization with patients' own pretransplantation PBL. Thirteen patients were studied 3 times or more, 2 patients were studied twice, and 1 patient was only studied once after grafting. We analyzed a total of 57 posttransplantation T cell cultures for the presence of Th cell activity against host and donor stimulator cells in a PLT assay (see the accompanying article). Proliferative responses of more than 5000 cpm were considered positive. Th cell activity against host cells was detected in 25 of a total of 57 antihost sensitized cultures (Table 1). Twenty-one of the host-reactive cultures did not proliferate against autologous donor cells, whereas only 4 cultures responded to host as well as donor cells. Thus, the

majority of all posttransplantation Th cell reactivities detected were host-specific.

Absence of *in vitro* generation of antihost-specific Th cell activities from unprimed donors All patients and donors studied here were mutually MLC-non reactive before grafting (data not shown). To investigate, however, whether donor antihost-specific Th cell activity could be generated by *in vitro* long-term culture, PLT assays were also performed with T cell lines derived from 12 marrow donors (see the accompanying article). In 8 of the 12 host-sensitized donor T cell lines, no Th cell activity was detected against either host or donor stimulator cells (Table 1). The 4 remaining donor T cell lines did proliferate against host-stimulator cells, 3 of which also responded against autologous donor cells and thus were not host-specific. One female donor, who was sensitized by pregnancies, showed proliferation against host but not donor cells. This response was considered host-specific.

Kinetics of the antihost-specific Th cell activities after transplantation The 25 cultures with host-specific Th cell activity (Table 1) were derived from 10 of 16 patients (Fig. 1). Six patients remained nonresponsive for at least one year (i.e., 2,3,4,6,12,13). The other 10 patients showed variation with respect to the magnitude and the kinetics of the antihost Th cell response. In some patients the antihost Th cell activities did not exceed levels up to 22,500 cpm (1,5,7,8,14), whereas in other patients levels of $45\text{--}100 \times 10^3$ cpm could be reached (9,10,11,15,16). Of the 10 responsive patients, 9 were evaluated during the first 3 months (1,5,7,9,10,11,14,15,16). In all of the latter patients the Th cell response was initiated within this period. In the other patient who was not evaluated during the first 3 months (8), antihost Th cells were detected immediately thereafter (day 97). In 6 cases the antihost Th cell activity peaked within the first 3 months (1,5,9,14,15,16), whereas in the other patients the responses increased until optimum levels

were reached between 6 and 12 months (8,10,11). After this, the antihost Th cell activity remained relatively stable as in patients 5, 9, and 10, but it could also gradually or abruptly decrease with time (1,8,14,16). The average log of the antihost Th cell activity found in the latest interval (12–25 months, 0.28 ± 0.38 cpm) was significantly lower than the averages found between 1.5 and 3 months (1.62 ± 0.35 cpm, $P=0.012$), between 3 and 6 months (1.45 ± 0.38 cpm, $P=0.038$), and between 9 and 12 months after BMT (1.32 ± 0.39 cpm, $P=0.047$).

Early regeneration of the Th cell activity against HLA alloantigens. To test the possibility that the early nonresponsiveness to host antigens as was observed in patients, 1, 2, 3, 4, 6, 12, 13, and 14 might reflect a general Th cell impairment (as a result of incomplete T cell reconstitution or immunosuppression), we analyzed the capacity of these patients' lymphocytes up to 9 months after grafting to mount a Th cell response against HLA alloantigens. For this purpose, a total of 20 samples derived from these patients were also sensitized against HLA mismatched stimulator cells and tested in the PLT assay (see accompanying article). All samples showed HLA alloreactive Th cell activity, except 1 sample from patient 13 (0–1.5 months, Table 2). Thus patients' samples which were nonresponsive against the host were generally not defective in exhibiting Th cell alloresponses.

Correlation between antihost-specific Th cell activities and the occurrence of acute GVHD. The patterns of antihost Th cell activities in patients without GVHD (Fig. 1A) were compared with the results obtained in patients developing acute GVHD (Fig. 1B) or acute, followed by chronic GVHD (Fig. 1C). Each of 5 patients with acute GVHD generated antihost Th cells; in 3 of these (9, 10, 11) high levels of activity ($>45 \times 10^3$ cpm) were reached. Th cells were also found in 2 of 6 patients without GVHD, and in 3 of 5 patients with chronic GVHD—2 of the latter reaching high levels of activity. The average log of the antihost Th cell activity in patients with acute GVHD (3.26 ± 1.23 cpm) was significantly higher than in patients without GVHD (-0.38 ± 0.96 cpm, $P=0.036$). The average log of the antihost Th cell activity in patients with chronic GVHD (0.38 ± 0.94 cpm) did not significantly differ from the averages found in patients with acute GVHD or patients without GVHD.

DISCUSSION

Host-directed proliferative T cell responses were commonly detected after HLA-identical BMT, in some cases as early as

TABLE 2. Tempo of regeneration of Th cell activity against major (HLA) alloantigens after BMT

Patient	Time after transplantation (in months)			
	0-1.5	1.5-3	3-6	6-9
1	60,800 ^a	— ^b	—	43,200
2	NT ^c	39,900	—	34,100
3	NT	—	74,300	88,100
4	—	—	73,500	—
6	—	—	94,000	83,100
12	19,800	42,000	42,100	36,200
13	900	33,000	40,200	NT
14	70,000	66,200	5,000	33,000

^a Response against specific stimulator cell (cpm); no responses were found against autologous donor cells (data not shown).

^b Blood sample not available.

^c Blood sample not tested.

day 22, and they persisted for at least two years. The most likely explanation for such reactivity was in vivo sensitization of donor lymphocytes against mH antigens present in the host. Consistent with the involvement of mH antigens, which are not stimulatory in primary responses, we showed that 11 of 12 donors tested prior to transplantation failed to generate an in vitro host-specific Th cell response. One female donor, however, probably in vivo-primed for common mH antigens by multiple pregnancies, developed in vitro host-specific proliferative activity (this study) as well as cytotoxic activity (see the accompanying article). Our main purpose was to investigate whether host-directed Th cells should be considered a possible risk factor in human GVHD. Although the evidence is still preliminary, the results favor this. All patients with acute GVHD developed antihost-specific Th cell activity compared with only one-third of the patients without GVHD. Statistically the response levels in both groups of patients were significantly different ($P=0.036$). As pointed out in our accompanying article, it was not possible to obtain further support for the role of antihost Th cells by analyzing these cells prior to the onset of GVHD, since the patients were lymphopenic. Yet antihost Th cells could be detected as early as 6, 11, 15, and 17 days after the diagnosis of GVHD (Fig. 1, B, C, patients 7, 15, 11, and both 10 and 16, respectively).

The experiments described here were carried out by using standard PLT assays. Other studies using the MLC technique failed to demonstrate proliferative antihost reactivities in patients with acute GVHD but were only successful in patients with chronic GVHD (18, 19). One possibility put forward to explain the latter results was immunologic "paralysis" from exposure to excessive host antigens in the acute phase of GVHD. However in view of our findings, prolonged in vitro exposure to antigen, such as in PLT, is probably required for adequate detection mH antigen-specific Th cells.

Although our current data suggest a possible role for a DTH-like mechanism, the presence of antihost Th cells in two patients without GVHD (1, 5) also indicates that the association with GVHD is not absolute. Maybe antihost Th cell activity in itself is not sufficient to induce GVHD. Alternatively, as was discussed for the presence of Tc cells, Th cells found in patients 1 and 5 might persist in an "anergic" state in vivo. Interestingly, these patients had received MTX as GVHD prophylaxis. In contrast to this, patients receiving CsA all displayed in vitro as well as "in vivo" antihost activity in this study. Yet, as is stated elsewhere, the evidence is too limited to speculate about the role of MTX versus CsA in the induction of tolerance.

On the other hand, if antihost Th cells are essential in GVHD pathogenesis, it remains puzzling why patients such as 12 and 13, with acute GVHD and subsequently chronic GVHD are nonresponsive for at least the period studied (2 years and 1 year, respectively). It is worthwhile to note that these latter 2 patients, although completely lacking antihost Th cells, had mounted a strong antihost cytotoxic response (see the accompanying article). Whether this observation is relevant to the presumed difference in effector cell mechanisms underlying the processes of acute and chronic GVHD (20) is unknown. In itself, such a discrepancy between Tc and Th cell responsiveness to mH antigens is a point of interest, and concerns the question of whether Tc and Th cells recognize different spectra of mH antigens. No evidence of this in man has been provided. In mice, however, the variable capacity of L3T4⁺ helper T cells

to mediate GVHD across mH antigenic barriers, in contrast to the less-variable capacity of Lyt2⁺ cytotoxic T cells (6-8, 10, 11) indeed suggests that L3T4⁺ cells may be limited to recognize only a very small number of mH antigens, perhaps far fewer than Lyt2⁺ cells. Another explanation could be that Th cells are overgrown in vitro by Tc cells. This appears unlikely, at least for patient 12, since several post-BMT T cell lines from this patient displayed an equal phenotype distribution of proliferative (CD4) and cytotoxic (CD8) cell markers (see the accompanying article). Furthermore, in a considerable number of T cell lines derived from other patients we were able to detect antihost Tc and Th cell activity at the same time (in this and the accompanying study). Finally, selective suppression (or elimination) of antihost Th cells may have been accomplished in vivo before the Th cell response actually had reached a critical stage in differentiation. This has been proposed to explain the phenomenon of immunodominance in the murine Tc cell response to mH antigens (21).

Evidently, the immunobiology of the mH antigen-specific Th cell response and its consequences in bone marrow grafting still need to be clarified. The current understanding, from both our studies, in agreement with animal models (5-12), is that multiple effector mechanisms might be involved in mH antigen GVHD but that one may predominate in a given transplant combination.

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