High frequencies of cytotoxic T cell precursors against minor histocompatibility antigens after HLA-identical BMT: absence of correlation with GVHD

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Summary:

Limiting dilution analysis was used to quantify the frequency of cytotoxic T cell precursors (CTLp) against minor histocompatibility (H) antigens induced by HLA-identical BMT. The development of CTLp was monitored serially in ten patients developing either acute (n = 3), acute and chronic (n = 4) or no (n = 3) GVHD. In blood samples of patients taken shortly after BMT (<100 days) a high frequency of anti-recipient CTLp was found (mean 1/3433). With time, this value decreased to become undetectable (<1/500 000) beyond 400 days. This occurred also in patients still suffering from chronic GVHD. In contrast, autologous BMT did not induce any measurable recipient-reactive CTLp at any time point after BMT. In the early phase of reconstitution after BMT the frequency of CTLp against allo HLA-antigens was measured in the same patients. The absence of a consistent increase of allo-specific CTLp indicates that the kinetics of CTLp against host minor H antigens does not merely reflect an overall changed cytolytic potential shortly after BMT.

These results indicate that: (1) HLA-identical BMT induces high frequencies of minor H antigen-specific CTLp detectable in the blood during the early phase of reconstitution, and (2) the frequency of recipient-reactive CTL measured in the peripheral blood is not an adequate parameter for GVHD. These data therefore challenge the clinical value of in vitro measurement of recipient-reactive CTLs in the peripheral blood after HLA-identical sibling BMT.

GVHD is still a major problem in allogeneic BMT, occurring in 45% of recipients of HLA-genotypically identical BM. Despite much effort, the effector mechanisms responsible for the pathogenesis of acute and chronic GVHD in humans remain obscure. To elucidate potential effector cells, several investigators have sought to define in vitro parameters measurable in the peripheral blood of recipients after BMT which would correlate with the incidence and severity of GVHD. Particularly, phenotypic markers such as NK cell number, CD4/CD8 T cell ratio or percentage of MHC class II + cells, as well as functional immunological parameters such as NK function, antibody dependent cell-mediated cytolysis and cytokyticity, PHA-responsiveness and cytotoxic T cell responses to allo-antigens were found not to discriminate between patients suffering from acute GVHD and those who did not. As GVHD does not occur after BMT performed in the absence of minor histocompatibility (H) differences or when mature donor T cells have been removed from the BM inoculum, donor T cells specific for recipient minor H antigens have also been considered as potential GVHD effector cells. Initial studies demonstrated the presence of recipient-specific CTLs in spleens of mice or in peripheral blood of individuals suffering from GVHD after MHC identical BMT, and uniformly implicated their role as effector cells in GVHD. Subsequent murine studies using BM inocula depleted of the Th or CTL population confirmed the role of minor H antigen specificCTLs in the induction of GVHD, although in some murine strains the Th cell population also appeared essential.

By contrast, in a recent study in humans, the presence of recipient-specific CTLs in PBL, as opposed to recipient specific T cells, proved to be insufficient and not required for GVHD pathogenesis after HLA-identical BMT. As in the latter study, host-antigen specific CTL lines were generated by repeated in vitro restimulation, the latter results only provided qualitative information on the presence of minor H antigen-specific CTLs. In a previous report we found that minor H antigen-specific CTLs could be quantified in PBL of a primed individual using a primary 7 days limiting dilution (LD) assay. We therefore have set out, using a LD assay, to quantify the anti-minor CTL response at several time points after minor H-disparate BMT in ten patients. Our aim was to ascertain whether the frequency of recipient-reactive cytotoxic T cell precursors measured in the blood would be a sufficient parameter for the incidence of GVHD after HLA-genotypically identical BMT.

Patients and methods

Patients

Ten patients received non-T cell depleted BM from their HLA-A, -B, -Cw, -DR identical, MLC non-reactive sibling donor. One patient who received
autologous BMT was also included. As conditioning patients received Cy (60 mg/kg/day × 2) and total body irradiation (8 Gy). To prevent acute GVHD all patients were given MTX during the first 3 months except patient 9 who received CsA. Acute GVHD was treated with prednisone or methyl prednisone. Chronic GVHD was treated with prednisone and/or azathioprine. Relevant information on the patients is summarized in Table I.

Blood samples
Heparinized blood samples were collected from recipients before and periodically after transplantation from their BM donors and from unrelated HLA typed individuals. PBL were isolated by Ficoll–Hypaque density gradient centrifugation and stored in liquid nitrogen to be used as responder cells in limiting dilution assays.

Limiting dilution analysis (LDA)
Seven concentrations of responder cells (RC) (range 312-4000/well) were set up in round bottom microwell plates in the presence of irradiated stimulator cells (SC) in 200 μl of RPMI 1640 supplemented with 15% pooled human serum and 20 U/ml rIL 2. Responder cells were either PBL of the BM donors of the BM recipients at several dates after BMT and of unrelated HLA mismatched individuals. Stimulator cells were Epstein-Barr virus (EBV) transformed B cell lines (5 × 103/well) (5000 cGy) of the BM recipient before BMT or of an unrelated HLA typed individual.

From each dilution, 24 wells were set up. After 5 days 100 μl containing 20 U/ml rIL 2 was refreshed and at day 7 the wells were assayed for cytotoxicity towards 104 PHA T cell blasts of the original stimulator in a standard 51Cr release assay. EBV B cell lines were never used as target cells to exclude as contribution of EBV antigen specific CTLp to the value measured.

Cultures were considered positive when showing lysis exceeding mean spontaneous release (24 wells containing no RC) plus 3 sds. LD wells never contained any nonspecific cytotoxicity of autologous (= responder) PHA blasts.

Statistical analysis
Frequencies of responding CTLp, 95% confidence intervals and goodness of fit were calculated using the minimum chi squared, maximum likelihood and Jackknife methods. All experiments shown had a p > 0.05 for linearity indicating single hit kinetics. Frequencies are referred to as high (>1/5000) low (1/100000-1/500000) or undetectable (<1/500000).

Results
Absence of correlation between frequencies of CTLp against minor H antigens and GVHD after HLA identical BMT
We set out to quantify the anti recipient CTL response induced against minor H antigens by HLA identical BMT and to monitor the kinetics of the anti minor CTLp frequency in time. PBL of BM recipients at two to four dates in the first 2 years after transplantation were analysed from three patients suffering from acute GVHD (Figure 1B) from four who developed additional chronic GVHD (Figure 1C) and from three patients without any GVHD complications (Figure 1A) (see Table I). The frequency of CTLp in each of these blood samples against recipient specific minor H antigens was determined in a primary in vitro LD assay using EBV B cells of the recipient before BMT as stimulator cells and testing the responding CTLp for minor H antigen specific lysis of the PHA T cells of the recipient before BMT. All blood samples of a single

<p>| Table I Clinical information concerning the patients |
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ANL: acute non lymphoblastic leukemia
1 Day of onset of acute GVHD
2 Grade of chronic GVHD (0: none 1: limited 2: extensive)
3 In contrast to patients 1-10 receiving autologous HLA identical BM control patient 1 received autologous BM
Quantitation of anti minor CTLp after BMT

Donor/recipient pair were always tested in the same experiment to exclude the kinetics to be influenced by inter experimental variation. As expected, no recipient-antigen-specific CTLp could be detected (<1/500000) in PBL of the unprimed donors (Figure 1A–C), with the exception of donor 9, whose PBL prior to BMT were already found to contain a frequency of 1/10000 CTLp against her HLA-identical sister (Figure 1C). As this female donor had been sensitized by pregnancies, this may explain the presence of anti-minor CTLp in her PBL before BMT. Surprisingly, blood samples of all patients available shortly after BMT (0–50 days), including those who did not develop acute GVHD (Figure 1A), were found to contain high frequencies of recipient-specific CTLp (Figure 1 range 1/1600–8065). In the subsequent period after BMT (50–100 days) the frequency remained high in most patients (1/4189–1/5491) except for patient 1 whose PBL contained only 1/35000 CTLp in the first sample available at 85 days (Figure 1C). Between 100 and 200 days after BMT, anti-minor H CTLp could still be detected in PBL of all BM recipient. However, values found in distinct individuals varied strongly (range 1/3086–1/110000) and seemed independent of the state of GVHD in a given patient (Figure 1A versus B versus C). All CTLp frequencies had uniformly declined to low values (1/11000–1/200000) between 200 and 400 days. Beyond 400 days CTLp frequencies were undetectable (<1/500000) in all patients, including those three patients still suffering from mild chronic GVHD at that point in time (Figure 1C). Only in patient 9, who had received presensitized BM, was a frequency of 1/80000 found. Thus, a high frequency of anti-recipient CTLp was found shortly after BMT declining in time to become low or undetectable beyond 200 days after

Figure 1 Kinetics of the anti recipient CTLp frequency after BMT. CTLp frequencies against recipient minor H antigens were measured in PBL of unprimed donors and of recipient at several dates after BMT. Results are shown of ten patients after HLA identical allogeneic BMT suffering from (A) no (B) acute or (C) acute + chronic GVHD and (D) one patient after autologous BMT. Reciprocal values of the frequencies and the 95% confidence intervals are indicated. pt = patient.
BMT in all ten patients analysed irrespective of their GVHD status.

Although nonspecific cytolytic activity was never observed in any of the CTLp limiting dilution cultures (see Materials and methods) we included an additional control to exclude the induction of recipient reactive CTLp being merely due to the process of BMT irrespective of minor histocompatibility differences between donor and recipient. As shown in Figure 1D, histocompatibility differences between donor and recipient are indeed essential for the development of CTLp after BMT because no anti-recipient CTL precursors (>1/500,000) could be detected at any time point in PBL of a recipient of autologous BMT (patient 11).

Frequencies of CTLp against allo HLA antigens are not affected by BMT

In 21 blood samples of 7 patients, the frequency of allo HLA antigen reactive CTLp was also determined. This was done for two reasons. Firstly, the recurrence of allo reactive CTL activity after BMT as measured in bulk CTL cultures remains a controversial issue. Secondly, to exclude the possibility that the observed kinetics of minor H antigen specific CTLp could reflect an altered overall cytolytic potential after BMT. The CTLp frequencies against allo HLA antigens were measured against EBV B cells of a healthy unrelated individual who had been selected on the basis of at least four HLA differences with all recipients. In addition, in each experiment the CTLp value between the same two HLA disparate individuals was measured. This value served as indicator value for inter experimental variation, known to be high in LD assays and which in our hands ranged between 1/3500 and 1/9090 in ten experiments (data not shown). The levels of allo CTLp frequencies varied between the distinct donor/recipient pairs, as was expected on the basis of distinct genetic backgrounds, immunization history and HLA (Figure 2). After BMT, in two patients a considerable reduction of the allo CTLp frequency was observed in the first blood sample, whereas at subsequent dates values resembled those of the donors before BMT (patients 1 and 8). In one patient a slight increase in the allo CTLp value was observed after BMT (patient 2). In the remaining four donor/recipient pairs tested, BMT did not significantly change the frequency of CTLp against allo HLA antigens (Figure 2, patients 4, 6, 10 and 11).

Discussion

In PBL of recipients of HLA identical BMT a high frequency of host reactive CTLs was observed during the first 100 days which declined in time to become undetectable beyond 1 year after BMT. The kinetics of anti minor CTLp followed the same trend in all ten recipients analysed, whether they suffered from moderate acute and/or chronic GVHD or not. No patients were analysed who developed severe acute or chronic GVHD Measurement of high frequencies in patients lacking any signs of acute GVHD and undetectable values in patients suffering from chronic GVHD late after BMT, most clearly indicated that the quantity of host reactive CTLp in the peripheral blood is not a predictive parameter for the occurrence of GVHD after HLA-identical BMT.

This observation can be interpreted in three ways. Firstly, the parameter measured may not indicate a specific change in the anti-minor CTL population after BMT but may merely reflect the changing lymphocyte composition during the hematopoietic reconstitution after BMT. Secondly, CTLs recognizing recipient-specific minor H antigens may not be relevant for the induction or pathogenesis of GVHD. Thirdly, minor specific CTLs may be involved in GVHD, but the incorrect in vitro parameter was measured. The first explanation that the high frequency of anti minor CTLp shortly after BMT is merely due to the rapid recurrence of CD8+ T cells, is contradicted by the fact that frequencies of CTLp to other antigens such as allogeneic HLA antigens as well as to mitogens are unaffected or even decreased in the first 3 months after BMT (Figure 2). Furthermore, the anti-recipient CTLp as measured here is shown to be undetectable after autologous BMT. The second explanation that CTLs reactive to host minor H antigens may be insufficient or not required for the development of GVHD would be compatible with our data. This would be in line with recent reports in the mouse where
T cell clones reactive to minor H antigens resulted in GVHD, whereas injection of cytotolytic T cell clones specific for other minor H antigens did not. However, these studies, in fact, do not exclude a contribution of CTLs to GVHD pathogenesis, but rather stress that involvement of anti-minor T cells in GVHD does not solely depend on their CTI/Th phenotype, but may also depend on their lymphokine production and the minor H antigen recognized.

A third interpretation is that anti-minor CTLs can be relevant in GVHD in vivo, however, that the in vitro measurement performed is not a relevant one. The blood may not be the appropriate site for monitoring those CTLs potentially relevant in GVHD. The observed kinetics of recipient-reactive CTLp in PBL could well be attributed to an initial peripheral expansion as a result of activation by residual host APC, followed by redistribution and migration out of the blood into the target tissues. Whereas this lymphocyte migration was demonstrated in the rat, thus far no systematic functional analysis of lymphocytes infiltrating GVHD-damaged organs has been performed in humans. Another argument for measurement of an inappropriate in vitro parameter may be that those CTLs with antigen specificities relevant in GVHD were not measured. The CTL population induced by BMT over a multiple minor H barrier followed by in vitro boosting using lymphoid cells may, as in the mouse, recognize only a limited number of ‘dominant’ minor H antigens. In a recent murine study it was questioned whether these minor H antigens, dominant in the induction of an in vitro CTL response, are indeed the ones relevant for the induction of GVHD in vivo. In addition, using a LD protocol with EBV-BLCL as stimulator cells, CTLs reactive with recipient minor H antigens expressed only on lymphoid cells or on both lymphoid and parenchymal tissues can be detected. However, CTLs reactive with tissue-specific minor antigens expressed only by the parenchymal cells of GVHD target organs are not detected using this assay, whereas they might strongly contribute to the local GVHD phenomena. The latter hypothesis would be compatible with the finding that the only two in vitro assays currently available with predictive value for the development of anti-minor GVHD, use skin cells as stimulator cells. Also, CTLs specific for the skin specific murine minor EPA-l have been shown to inflict GVHD-like tissue destruction when injected in vivo. Should the latter assumption be correct, i.e. should the CTLs recognizing relevant GVHD minor not have been detected using this assay, then the question arises as to what the in vivo function of the high frequent host-reactive CTLs detected in PBL shortly after BMT could be. One possibility would be that these CTLs define minor H antigens restricted to the hematopoietic lineage and therefore could be selectively involved in graft-versus-leukemia reactivity. Whereas no patients suffering from leukemia relapse were included in this study, van Els et al found that within a panel of 16, only the two patients developing a relapse did not have measurable anti-recipient CTLs.

In conclusion, a limiting dilution method is described for quantitation of minor H antigen-specific CTL precursors induced by HLA-identical BMT. The frequency of CTLp against recipient antigens in the peripheral blood is shown not to correlate with the development of GVHD in vivo. These results challenge the clinical value of in vitro assays measuring potential GVHD effector cell populations in the peripheral blood. Furthermore, it raises the question of what the host-reactive CTLs present in high frequencies shortly after BMT might do in vivo.

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