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Acquired tolerance for minor histocompatibility antigens after HLA identical bone marrow transplantation

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Abstract

Skin tissue of a healthy chimera 7 years after HLA identical bone marrow transplantation was found to express a minor histocompatibility (mH) antigen against which cytotoxic T lymphocytes (CTLs) had been discovered at a time of acute graft-versus-host disease (aGVHD). We were prompted to investigate the apparent tolerance to this persistent mH antigen and used limiting dilution analysis to monitor *in vitro* anti-host CTL responses in time after bone marrow transplantation. A high anti-host CTL precursor frequency was found during acute GVHD, declining in time until beyond detection level in the healthy chimera 7 years after transplantation. In this case report (i) CTL precursor frequencies are used for the first time to monitor *in vitro* tolerance induction to persistent host mH antigens after HLA identical BMT in man; and (ii) it is shown that LDA may be a potential tool for quantification and specificity analysis of CTL responses to mH antigens.

Introduction

The mechanisms underlying induction and maintenance of tolerance after allogeneic bone marrow transplantation (BMT) are still unclear (1) Although tolerance after MHC-identical BMT has been addressed by some authors (2–6), the issue is complicated because analysis of reactivity to minor histocompatibility (mH) antigens is hampered by the absence of a primary *in vitro* graft-versus-host response before transplantation (5) In man, it is established that in patients having received HLA identical bone marrow, mH antigen reactive cytotoxic T lymphocytes (CTLs, 6–8), T_h cells (9), and specific (5) as well as non-specific (10) suppressor cells can be detected However, whether, and if so how, these respective cell types can account for the development of graft–host tolerance is controversial (3,4)

Here we report on a patient who enabled us to address the issue of acquired tolerance after HLA genotypically identical but mH antigen mismatched BMT His peripheral blood leukocytes (PBL) studied *in vitro* at a time of acute graft-versus-host disease (aGVHD) contained host reactive CTLs recognizing a self HLA class I restricted mH determinant HA-3 (6), detectable not only on the patient's pre-BMT PBL but also on his post-BMT skin-derived keratinocytes and fibroblasts. Seven years post-BMT, this full donor chimera was in good health and must have acquired tolerance to host mH antigens such as HA-3, shown to be persistently expressed in patient skin tissue after BMT We were prompted to investigate *in vitro* anti-host CTL reactivity in time using limiting dilution analysis (LDA) and found that (i) CTL responses directed to host mH antigens after BMT could be measured in a primary *in vitro* assay, (ii) split-well analysis of primary anti-host LD cultures allowed analysis of the repertoire of mH antigen reactive CTLs triggered by BMT, and (iii) the anti-host CTLp frequency in this patient, high during aGVHD, declined in time to beyond detection level at 7 years post-BMT, and therewith paralleled the *in vivo* state of tolerance.

Methods

Patient

A 29 year old male AML patient received non-depleted bone marrow of his HLA identical (HLA-A1,-A11,-B8,-B60,-Cw3,-DR2, -DR3), MLR-negative, non-transfused brother Rapid engraftment followed by aGVHD grade II of primarily gut and liver (diagnosed day 26) was treated with decreasing amounts of prednisone Graft-versus-host reactions revived at day 85 involving skin, gut, and liver After renewed prednisone treatment (days 124–134) graft-versus-host reactivities definitively disappeared, this patient

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is at present in apparently good health 9 years post-BMT FACS analysis of the patient's PBL revealed high frequencies of monocytes shortly post-BMT 71% on day 24 and 51% on day 141 An inversed CD4/CD8 ratio was found (day 24, 0 28) due to both an increase in $CD8^+$ and a decrease in $CD4^+$ T cells At 7 years post-BMT the inversed ratio persisted (0 38) whereas B cell and monocyte values had normalized The presumed state of full donor chimerism could not be confirmed by restriction fragment length polymorphism because of lack of discriminative markers However, the absence of any host-derived cells in the B and T cell compartment could be shown as early as day 24 post-BMT by using a cellular marker (not shown)

Keratinocyte and fibroblast cultures

Keratinocytes were isolated from patient, donor, and third party arm skin biopsies, and cultured as previously described (11) Wells of microtiter plates were coated with layers of cultured keratinocytes and, after ^{51}Cr-labeling, used as adherent target cells in the cell-mediated lympholysis (CML) assay (11) The patient's skin-derived fibroblasts were cultured in DMEM (Flow) + 5% FCS and were also used as adherent targets in CML (12)

Host-reactive T cell lines

PBL at several time points after BMT were repeatedly stimulated with patient post-BMT PBL to yield anti-host CTL lines as described elsewhere (6) From a day 24 post-BMT CTL line, an anti-host CTL clone (5Ho11) was derived, which showed HLA-A1 restricted recognition of a non-HLA determinant designated as HA-3 (6) T cell lines generated in this way from 7 years post-BMT PBL contained no cytotoxicity towards the patient's pre-BMT phytohemagglutinin (PHA) blasts (not shown)

Limiting dilution analysis

Graded numbers of responder cells ($156-4 \times 10^4$ cells/well) were set up 24-plicate in round-bottomed microtiter plates in the presence of irradiated stimulator cells in 200 μl of culture medium (RPMI 1640, 15% human serum) with 20 U rIL-2/ml (13) Stimulator cells were either polymorphonuclear cells (PMNC) (5×10^4/well rad) or Epstein – Barr virus lymphocyte cell line (EBV-LCL) (5×10^3/well, 5000 rad) After 5 days 100 μl containing 15 U rIL-2/ml was refreshed and at day 7 the wells were assayed for cytotoxicity towards PHA blasts in a standard ^{51}Cr release assay Cultures were considered positive when showing lysis exceeding mean spontaneous release (24 wells) plus 3 SD LD wells never contained any non specific cytotoxicity of autologous (= responder) PHA blasts Frequencies of CTLp and 95% confidence intervals were calculated using the minimum χ^2, maximum likelihood, and jacknife methods In some experiments the wells were split and 50% of the wells' contents was assayed for cytotoxicity Host reactive wells were expanded using present EVB-BLCLs (10^5/ml, 5000 rad) and autologous donor PBL (10^6/ml, 3000 rad) in culture medium with 2% HP IL 2 (Biotest) One or two stimulation cycles were needed to obtain enough of each oligoclonal CTL culture to test panel reactivity and reactivity to host skin cells in a CML assay

Results

Minor antigen HA-3 specific lysis of host skin

The HA-2 specific, HLA-A1 restricted CTL clone 5Ho11 obtained from the patient's PBL during aGVHD was tested for recognition

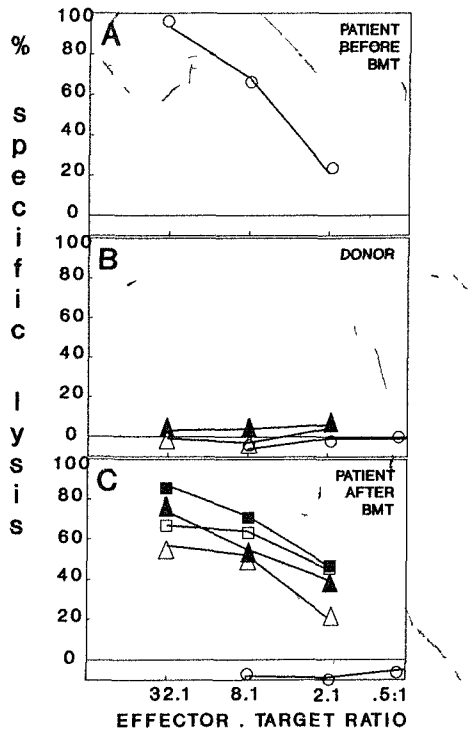

Fig. 1. Split chimerism for the HA-3 antigen after BMT The HA-3 specific HLA A1 restricted CTL clone 5Ho11 was tested in a 4 h ^{51}Cr release assay for lysis of PHA blasts (○), keratinocytes (Δ ▲ + 24 h 250 U/ml rIFN γ), and fibroblasts (□, ■ 24 h 250 U/ml rIFN γ) of the HLA identical donor (B), and the patient before (A) and after BMT (C)

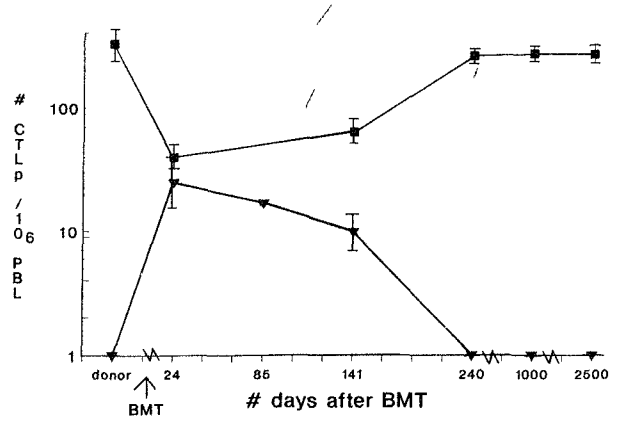

Fig. 2. Development of CTLp frequencies against mH and allo HLA antigens in time after HLA identical BMT CTLp frequencies represent mean values + SE of five experiments and were measured using PBL of the patient before BMT (▼) and PBL of an unrelated fully HLA mismatched individual (■)

of donor and patient post-BMT PHA blasts, keratinocytes, and fibroblasts, and of the patient's post BMT PHA blasts Figure 1 shows that the patient's PBL, expressing HA-3 before HLA identical BMT (A), had become HA-3 negative after BMT (C), as fits with complete hematopoiesis from the HA-3 negative, HLA identical donor bone marrow (B) In contrast, the patient's post-BMT keratinocytes and fibroblasts, with or without previous incubation with rIFN γ, were lysed by the anti-HA 3, HLA-A1

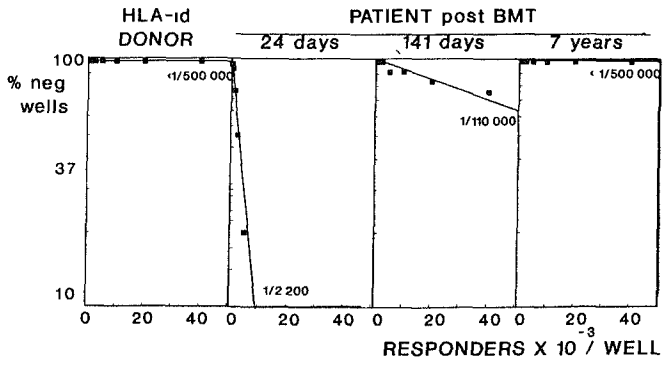

Fig. 3. Measurement of mH antigen reactive CTL precursors after BMT PBL of the donor and the patient post-BMT (days 24 and 141, and 7 years) were tested as RC in LDA with patient post BMT EBVL-LCL as SC The percentage negative of 24 wells is presented and the calculated CTLP frequencies are given

restricted CTLs (C), thus indicating the persistent expression of the mH antigen after BMT

Host and alloreactive CTL precursor frequencies after BMT

LD cultures were set up using several patient's post-BMT samples and unprimed donor PBL as responder cells (RC) and patient pre-transplant PBL as stimulator cells (SC) The same RC were also stimulated with PBL of an unrelated HLA mismatched individual In four of five experiments we observed the same trend of anti-host CTLp frequencies in time (Fig 2) In the unprimed donor PBL a very low (1/200,000 – 500,000) or undetectable frequency (< 1/500,000) against his HLA identical brother was found After BMT repopulating donor cells revealed a significant frequency of host specific CTL precursors The frequency of mH antigen reactive CTLp rapidly declined in time and could no longer be demonstrated in blood samples of 240 days – 9 years after BMT (Fig 2) The CTLp frequency against allo-HLA antigens revealed the reverse trend, being severely decreased shortly post-BMT, restoring in time to approach the allo-CTLp frequency found for the HLA identical donor (Fig 2) Since the absolute values of mH antigen reactive CTLp frequencies measured were low (< 1/29,000), we also used EBV-LCL to measure anti-host CTLp frequencies after BMT The same development in time was observed with significantly higher frequencies (Fig 3)

Specificity of host-reactive CTLs after BMT

From one LD experiment using day 24 post-BMT PBL as RC and patient post-BMT PBL as SC, wells were split, screened for cytotoxicity of host PHA blasts and expanded Of 33 oligoclonal CTL lines obtained from positive LD wells, 26 contained host specific CTLs (i e lysis > 15% of patient post-BMT and < 1% of donor PHA blasts) The recognition pattern on PHA blasts from eight unrelated individuals each sharing one HLA antigen with the responder cells are shown in Fig 4 The panel analysis (n = 16) indicated that recognition could occur in the context of all self HLA class I antigens (Fig 4C), although HLA-A1 appeared the predominant restriction antigen (Fig 4A and B) Testing cytotoxicity towards pairs of HLA-identical HLA-A1 positive, HA-3 incompatible (n = 5), and HA-3 compatible (n = 3) siblings indicated that a large fraction of the HLA-A1 restricted CTL cultures might contain HA-3 like reactivity (one HA-3 incompatible pair is shown in Fig 4A)

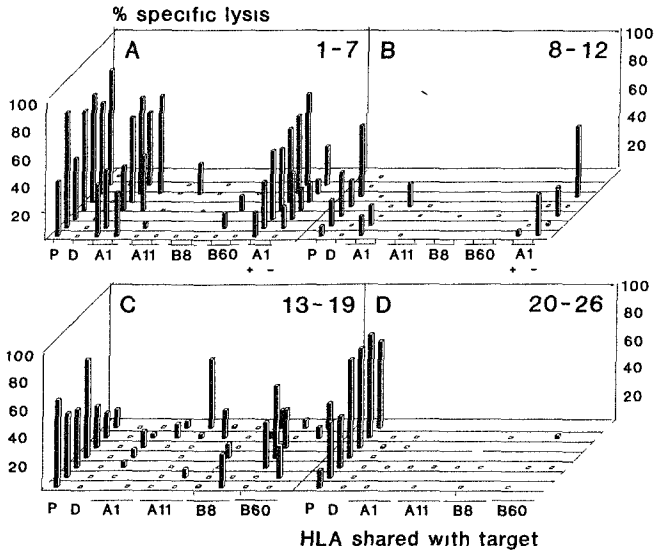

Fig. 4. Panel reactivity of cytolytic host reactive LD cultures Twenty-six oligoclonal CTL lines obtained from positive LD wells containing 2.5 – 40 × 10^3 of day 24 PBL were tested for lysis of a panel of PHA blasts, including patient pre-BMT (P), donor (D), eight unrelated target cells sharing either HLA-A1, -A11, -B8 or -B60, and two target cells derived from an HLA-A1$^+$, HA-3 disparate (indicated +, –) sibling pair CTL lines are presented according to possible HLA restriction A, B HLA A1, C other, D no panel reactivity Values of lysis observed at E:T = 30 are shown

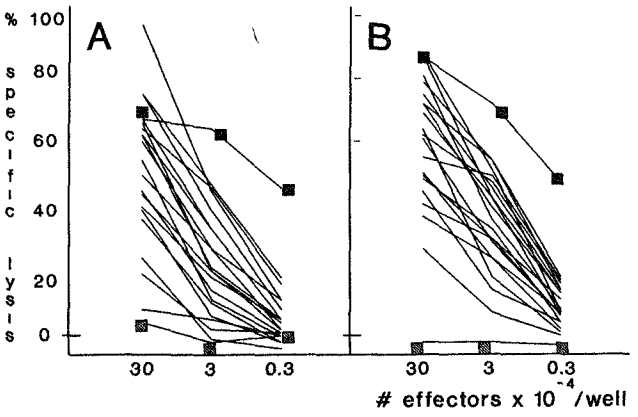

Fig. 5. Eighteen CTL lines obtained from day 24 LD cultures were tested for recognition of the patient's post-BMT cultured dermal fibroblasts (A untreated, B 25 U/ml rIFN-γ, 48 h) in a 4 h ^{51}Cr release assay LD wells had been expanded 2 weeks before usage as effectors (—) The HA-3/HLA-A1 specific CTL clone 5Ho11 (■) served as a positive and a HLA-B7 specific CTL line (▨) as a negative control

Of the 26 CTL lines obtained from LD wells, 18 were also tested for reactivity towards the patient's post-BMT dermal fibroblasts All CTL lines to some extent lysed IFN-γ treated as well as untreated fibroblasts (Fig 5), indicating that of all *in vitro* expanded anti-host CTL precursors, reactivity is directed towards determinants which are expressed on the patient's non-lymphoid cells after BMT Additionally, CTL lines with clear HLA-A1 restriction proved capable of lysis of unrelated HLA-A1, HA-3$^+$ keratinocytes No cytotoxicity toward donor keratinocytes was found for any of the CTL lines (data not shown)

Discussion

PBL of patients after HLA-identical BMT are known to contain CTLs reactive to host mH antigens present on patients' pre-transplant PBL (6,8) The observation that skin cells of a healthy full donor chimera after HLA identical BMT expressed a patient specific mH antigen prompted us to monitor tolerance to host mH antigens *in vitro* In this patient we for the first time quantified graft-versus-host CTL reactivity in a primary *in vitro* assay using LDA A relatively high anti-host CTLp frequency was found during aGVHD, which gradually disappeared in time and became undetectable in PBL of the healthy patient after BMT (Fig 2a) By contrast, the CTLp frequency against allo-HLA antigens was found to be decreased shortly after BMT (Fig 2) This is consistent wiht earlier data, indicating a compromised T cell response to allo-HLA antigens after BMT (14) The striking relative predominance of anti-host CTLs shortly after BMT might be explained by preferential clonal expansion of these donor T cells which are activated by host antigens presumably on residual BM derived host antigen presenting cells (APC) (2) An indication of the nature of the antigens giving rise to this high anti-host CTLp frequency was obtained by analyzing host reactive LD cultures for HLA restriction (Fig 4) and tissue recognition (Fig 5) Recognition of the patient's post-BMT dermal fibroblasts *in vitro* (Fig 5) indicated that all CTL precursors detected in PBL have mH antigen specificities which enable them to interact with non-lymphoid tissue of host origin *in vivo* After the initial 5 months of aGVHD this patient is disease free, having a normalized allo HLA CTLp frequency, whereas the anti-host CTLp frequency could no longer be measured (Figs 2 and 3) This *in vitro* unresponsiveness to host antigens could reflect an *in vivo* state where host reactive T cells have been deleted or, alternatively, have been rendered inactive via specific or non specific suppressor mechanisms The presence of suppressor cells cannot be ruled out merely on the basis of LD analysis following single hit kinetics (15) We did not perform any *in vitro* suppressor cell assays and cannot discriminate between anergy, active suppression, or clonal deletion of host reactive cells in this patient Studies in murine radiation chimeras have suggested a need for either specific (3,4) or non-specific (as reviewed in 4) suppressor cells to maintain graft – host tolerance after transplantation across minor histocompatibility barriers In man, a role for antigen specific suppressor cells was suggested in an early study by Tsoi *et al* (5), who demonstrated a correlation between the presence of suppressor cells and long-term graft – host tolerance However, it should be stressed that, whatever is the underlying mechanism in this patient, we do not know whether the observed tolerization of the anti-host CTL response *in vitro* is causally related to the development of graft – host tolerance *in vivo* Using polyclonal cell cultures, van Els *et al* (7) showed in a study comprising 16 patients that the absence of host reactive CTLs was not sufficient to ensure long term graft – host tolerance We are currently investigating a panel of patients in order to settle the question whether the anti-host CTLp frequency is correlated with the *in vivo* state of graft – host reactivity after HLA identical BMT

Under the assumption that this is the case, we would like to propose a hypothetical mechanism for tolerance induction to HA-3 and other persistent mH antigens in this patient Keratinocytes, fibroblasts, and many other so-called 'non classical' APC (16) have been found to have no or little capacity of T cell activation, even after IFN γ induced HLA class II expression (17,18) HLA class II expressing keratinocytes (also found in aGVHD-affected skin, 16) were even found to tolerize hapten-specific T cell clones *in vitro* (19) In the full donor chimera studied here non-hematopoietic host tissues provide the only source of host mH antigens after BMT Indirect presentation of antigenic fragments to donor T cells by donor APC which have bound and reprocessed shed host antigen could occur after BMT However, though presentation via this mechanism to donor T cells may very well take place for class II restricted exogenous products, it is less likely to occur in the case of MHC class I restricted endogenous mH antigens Direct presentation of host mH antigen by parenchymal host tissues functioning as inadequate APCs could result in tolerization of anti-host CTLs induced during BM engraftment According to this hypothesis, the induction of long-term graft – host tolerance versus reactivity after BMT across minor histocompatibility barriers would depend on the tissue distribution of the mH antigens in question We are currently investigating the tissue distribution of human mH antigens

Finally, from a methodological point of view this case study reveals that it is possible to use LD methods to study CTL responses to mH antigens triggered by BMT If generally applicable, this would mean that the repertoire of host reactive CTLs could be analyzed systematically and quantitatively and perhaps it would even become possible to distinguish 'major' mH antigens, i e those that trigger a strong T cell response *in vivo*

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Abbreviations

aGVHD	acute graft versus host disease
APC	antigen presenting cell
BMT	bone marrow transplantation
CML	cell mediated lympholysis
CTL(p)	cytotoxic T lymphocyte (precursor)
EBV LCL	Epstein – Barr virus lymphocyte cell line
LDA	limiting dilution analysis
mH antigen	minor histocompatibility antigen
PBL	peripheral blood leukocytes
PHA	phytohemagglutinin
PMNC	polymorphonuclear cells
RC	responder cells
SC	stimulator cells

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