ELSEVIER

Journal of Immunological Methods 293 (2004) 51–59

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Research paper

Towards functional transplant donor matching by measurement of granzyme A and granzyme B production levels

Brigitte Kircher[a,*], C. Erik Hack[b], Anne M. Dickinson[c], Xiao N. Wang[c], Machteld Oudshoorn[d], Annemarie Sachs[d], Angela Wolbink[b], Dietger Niederwieser[a,e], Günther J. Eibl[f], Hans C. van Houwelingen[g], Els Goulmy[d]

[a]*Laboratory for Tumor and Immunobiology, Department of Hematology and Oncology, Innsbruck Medical University, Anichstrasse 35, 6020 Innsbruck, Austria*
[b]*Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands*
[c]*University Department of Hematology, School of Clinical and Laboratory Sciences, University of Newcastle upon Tyne, United Kingdom*
[d]*Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands*
[e]*Division of Hematology and Oncology, Leipzig University Hospital, Leipzig, Germany*
[f]*Institute for Biostatistics and Documentation, Medical University Innsbruck, Innsbruck, Austria*
[g]*Department of Medical Statistics, Leiden University Medical Center, Leiden, The Netherlands*

Received 23 September 2003; received in revised form 29 June 2004; accepted 30 June 2004
Available online 21 August 2004

Abstract

Graft-versus-host disease (GvHD) can be a major complication after allogeneic stem cell transplantation (SCT) especially when donor and recipient are unrelated. The latter serious complication, together with the growing number of available unrelated stem cell donors, demand a simple in vitro assay for functional stem cell donor selection. Activated donor cytotoxic T lymphocytes (CTLs) and natural killer cells produce granzymes (Gr) that are involved in the pathogenesis of GvHD. We measured granzymes A and B (GrA and GrB) production levels in the supernatants of 96 h pretransplant mixed lymphocyte cultures (MLC) of 26 sibling and 31 unrelated patient/donor pairs by enzyme-linked immunosorbent assay (ELISA). In detail, the GrA and GrB production levels from a selected cohort of 37 potential patient/donor pairs were correlated with relative responses (RR) of MLC and with human leukocyte antigen (HLA) class II mismatches and with the development of acute GvHD in a second, consecutive cohort of 20 sibling SCT recipients.

In vitro measurement of GrA and GrB production levels significantly correlated with the RR of pretransplant MLC (r=0.492, $p \leq 0.01$ and r=0.853, $p \leq 0.01$, respectively) and increased with the number of HLA class II mismatches between patient and donor. Pretransplant GrA production levels were significantly associated with the in vivo development of acute GvHD grades II–IV in patients transplanted with an HLA-identical sibling donor ($p \leq 0.001$). In conclusion, in vitro GrA and GrB production levels can

Abbreviations: CTLs, cytotoxic T cells; ELISA, enzyme-linked immunosorbent assay; Gr, granzymes; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; MLC, mixed lymphocyte culture; mRNA, messenger ribonucleic acid; NK, natural killer cells; PBMC, peripheral blood mononuclear cells; RR, relative response; SCT, stem cell transplantation.

* Corresponding author. Tel.: +43 512 504 23337; fax: +43 512 504 23338.
E-mail address: brigitte.kircher@uklibk.ac.at (B. Kircher).

doi:10.1016/j.jim.2004.06.024

be measured by a quantitative and sensitive ELISA. This novel and simple method may be used for functional selection of unrelated stem cell donors and for the identification of patients who are at risk for acute GvHD grades II–IV.

Keywords: Granyzme A; Granzyme B; ELISA; MLC supernatant; HLA class II mismatch; GvHD

1. Introduction

Graft-versus-host disease (GvHD) is a frequent complication affecting the outcome of stem cell transplantation (SCT; Deeg and Storb, 1984). Optimal histocompatibility between donor and recipient reduces the risk for the development of GvHD (Beatty and Henslee-Downey, 2000; Petersdorf et al., 1998; Sasazuki et al., 1998). However, the current in vitro assays applied for stem cell donor selection are laborious, not always consistent and are costly (Mickelson et al., 1993; Tiercy et al., 1993; Fussel et al., 1994; Schwarer et al., 1994; Healey and Schwarer, 2002). Moreover, with the still growing numbers of new human leukocyte antigen (HLA) allelic determinants, the optimal donor choice is becoming more difficult. We therefore searched for an alternative assay reflecting immune effector activities involved in GvHD pathogenesis.

Donor-derived cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are important mediators in the destruction of recipient's tissues and cells after SCT. The effector cells exert their cytolytic function through two major pathways (Russell and Ley, 2002). One is via an apoptotic mechanism which involves the pore-forming protein perforin and granulocyte-associated serine proteases (granzymes A and B (GrA and GrB)). It is regarded as the most important effector function of $CD8^+$ T cells and NK cells (Lieberman, 2003). The other cytolytic pathway is by the interaction of the Fas/APO-1 molecule with its ligand (FasL). GrA and GrB as well as perforin-expressing lymphocytes can be detected in biopsies from patients rejecting heart transplants and in the skin lesions of GvHD patients (Clément et al., 1991a; Griffiths et al., 1991; Higaki et al., 2001), and their expression can be correlated with the severity of acute lung (Clément et al., 1994) and liver rejection (Kuijf et al., 2002). Soluble Fas and FasL levels are increased during acute GvHD (Liem et al., 1998; Kanda et al., 1998).

In this study, we measured the production levels of GrA and GrB in supernatants of pretransplant patient/donor mixed lymphocyte cultures (MLC) by enzyme-linked immunosorbent assay (ELISA) and correlated the GrA and GrB production levels with the relative response (RR) of MLC, with HLA class II mismatches between patients and donors and with the development of acute GvHD.

2. Methods

2.1. Preparation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained after informed consent from donors and patients after treatment for their disease but before any conditioning for SCT. PBMC were separated from heparinized venous blood by density gradient centrifugation (Axis-Shield PoC, Oslo, Norway). Cells were washed twice and resuspended in culture medium which consists of RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 2 mM/l L-glutamine (Gibco), 1% antibiotics (Gibco) and 20% heat-inactivated human serum.

2.2. MLC

Donor PBMC (5×10^4) were cocultured with 5×10^4 irradiated (20 Gy) patient PBMC in 200 μl culture medium at 37 °C in a humidified 5% CO_2/95% air atmosphere. On day 4, supernatants for GrA and GrB measurements were harvested from each well and stored at −20 °C until use. Thereafter, cells were exposed to 2 μCi of ^{3}[H]thymidine (40–60 Ci/mmol, Amersham, Arlington Heights, IL) for 12–16 h, harvested and ^{3}[H]thymidine uptake was measured in a liquid scintillation counter (Beckmann, Galway, Ireland). Results are expressed as median counts per minute of triplicate values. The percent (%) RR of the

MLC was calculated as follows: (test MLC−autologous MLC)/(reference value−autologous MLC)× 100. The reference value is the median response to stimulation by unrelated controls.

2.3. HLA-typing

All patients and donors were typed by serology for HLA class I and class II antigens. The polymerase chain reaction (PCR)–SSP technique was applied for high resolution HLA-A, -B and -C typing using commercially available HLA-A and -B primersets (Dynal, Oslo, Norway) and a locally made HLA-C primerset. HLA class II genotypes were determined by PCR amplification with sequence-specific primers for HLA-DRB1, 3, 4, 5 and -DQB1. HLA-DPB1 typing was performed with PCR-sequence-specific oligonucleotides.

2.4. ELISA for GrA and GrB

Purification of the monoclonal antibodies (mAb) and the ELISA for GrA and GrB were performed, as described (Spaeny-Dekking et al., 1998). Briefly, microtiter plates were coated with 0.5 μg/ml mAb GB 29 (for GrA) or GA 11 (for GrB) for 16 h at 4 °C. Residual binding sites were blocked by a 45-min incubation with PBS/2% (v/v) cow milk. Samples were diluted in 40 μg/ml hyaluronidase (Sigma, St. Louis, MO) in high-performance ELISA buffer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and incubated in the plates for 1 h. After a further incubation with biotinylated mAb GB 28 (for GrA) or GA 10 (for GrB) in 1% (v/v) normal mouse serum, streptavidin-polymerized horseradish peroxidase (Department of Immune Reagents, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) was added to the plates for 30 min. The reaction was visualized by incubation with 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) and 0.003% (v/v) H_2O_2 in 0.11 M sodium acetate buffer (pH 5.5). After stopping the reaction by adding an equal volume of 2 M H_2SO_4, absorbance at 450 nm was determined by a Titer-Tek Multiscan plate reader (Labsystems, Helsinki, Finland). The Gr ELISA is now commercially available (Pelikine compact human GrA and GrB ELISA kit, Sanquin Blood Supply Foundation, Amsterdam, The Netherlands).

2.5. Statistical analysis

Descriptive statistics are given as median and as 25–75% percentile range. Spearman's correlation coefficient is used to assess the mutual correlation of GrA, GrB, RR and the total number of HLA class II mismatches. Mann–Whitney test is used to compare the levels of GrA, GrB and RR between subgroups defined by HLA mismatch or GvHD.

To avoid problems in the statistical analysis caused by the intrapair correlation of the measurement the "average" value of GrA, GrB and RR in each pair was computed and used for further analysis. In the light of skewness of the data, the geometric mean was used as an "average" for GrA and GrB.

To handle the zero values of RR, we first added 1% to all RR measurements, then took the geometric mean and finally subtracted 1% again to bring it back to the original scale.

3. Results

The first cohort comprising 31 unrelated and 6 sibling patient/donor pairs was selected to measure GrA and GrB production levels in the supernatants of pretransplant MLC and to correlate with the RR of MLC and with the number of HLA class II mismatches. From this cohort of patient/donor pairs, only one combination was actually selected for SCT. In a separate, consecutive cohort of 20 HLA-identical sibling patient/donor pairs, wherein all patients where transplanted, GrA and GrB production levels were measured in the supernatants of pretransplant MLC and correlated with the development of acute GvHD.

3.1. Correlation of GrA and GrB production levels with RR of MLC

MLC from each patient/donor pair were established in graft-versus-host and host-versus-graft direction. In both latter directions, the RR of MLC and the GrA and GrB production levels in the supernatants of MLC were determined.

Fig. 1 shows the correlation of the median GrA and GrB production levels of the 37 patient/donor pairs with the RR of the MLC. GrA and GrB production levels significantly correlate with each other (r=0.499, $p \leq 0.01$, Fig. 1a) and with the RR of the MLC (r=0.492, $p \leq 0.01$, Fig. 1b and r=0.853, $p \leq 0.01$, Fig. 1c, respectively).

3.2. Correlation of GrA and GrB production levels with the number of HLA class II mismatches

From the 37 patient/donor pairs studied, 6 sibling and 4 unrelated patient/donor pairs did not display any mismatch in HLA class II antigens. Of the 27 patient/donor pairs displaying HLA class II mismatches, 5

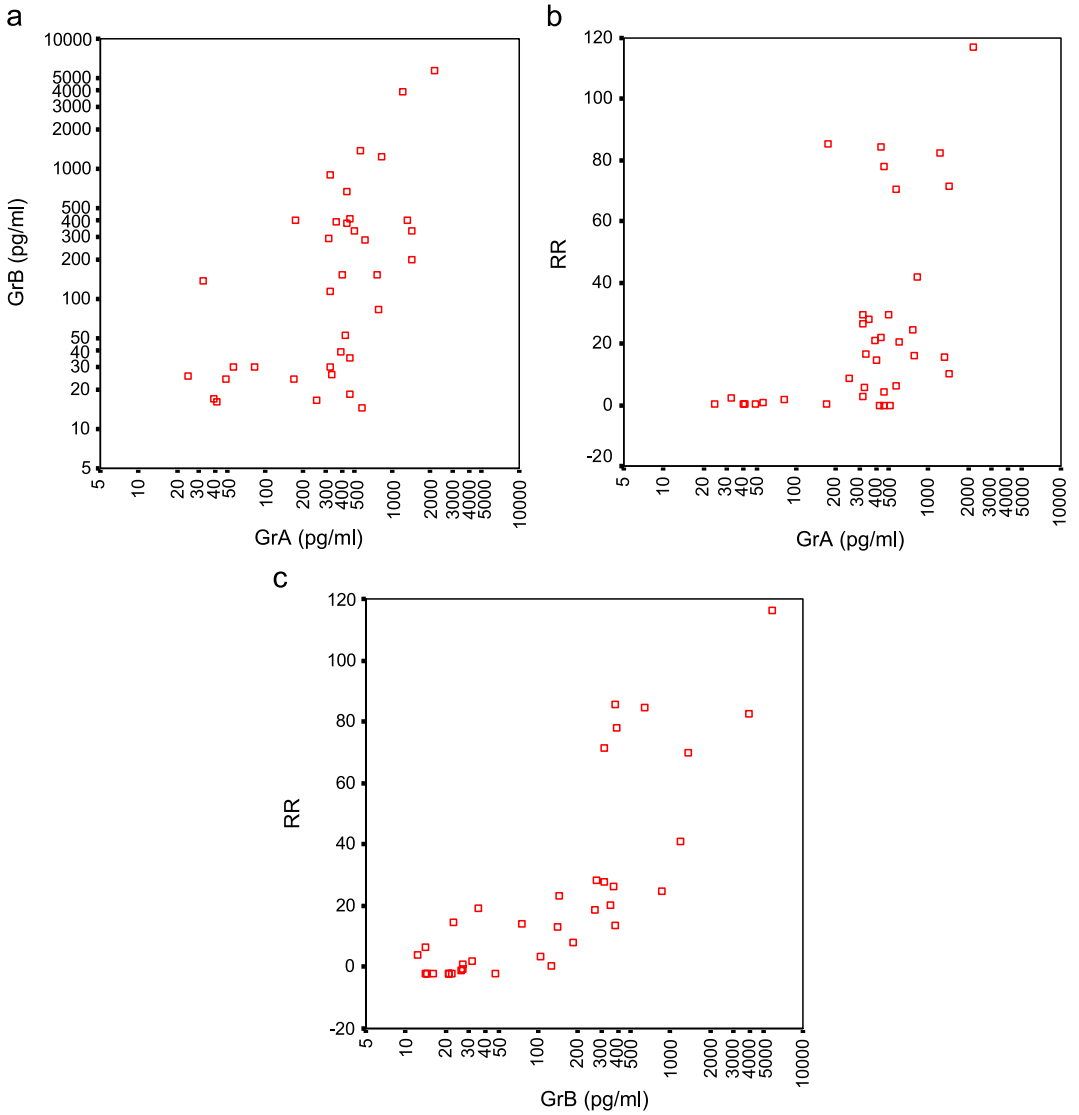

Fig. 1. Correlation of GrA and GrB production levels with each other and with the RR of the MLC. The results of the first cohort of patient/donor pairs comprising 31 unrelated and six sibling patient/donor pairs are shown. GrA and GrB production levels are depicted on a logarithmic scale. (a) Correlation of GrA production levels with GrB production levels. (b) Correlation of GrA production levels with RR of the MLC. (c) Correlation of GrB production levels with RR of the MLC.

patient/donor pairs showed one HLA class II mismatch (either HLA-DRB1, -DQB1 or -DPB1), 11 patient/donor pairs two HLA class II mismatches, 7 patient/donor pairs three HLA class II mismatches and 4 patient/donor pairs four HLA class II mismatches.

Fig. 2 shows the correlation of the GrA and GrB production levels with the number of HLA class II mismatches (r=0.579, $p \leq 0.01$ and r=0.650, $p \leq 0.01$, respectively) and the correlation of the RR of MLC with the number of HLA class II mismatches (r=0.798, $p \leq 0.01$). In detail, the median GrA production levels is 66 pg/ml (25–75% percentiles: 38–434 pg/ml) in supernatants of MLC of the patient/donor pairs without HLA class II mismatches and

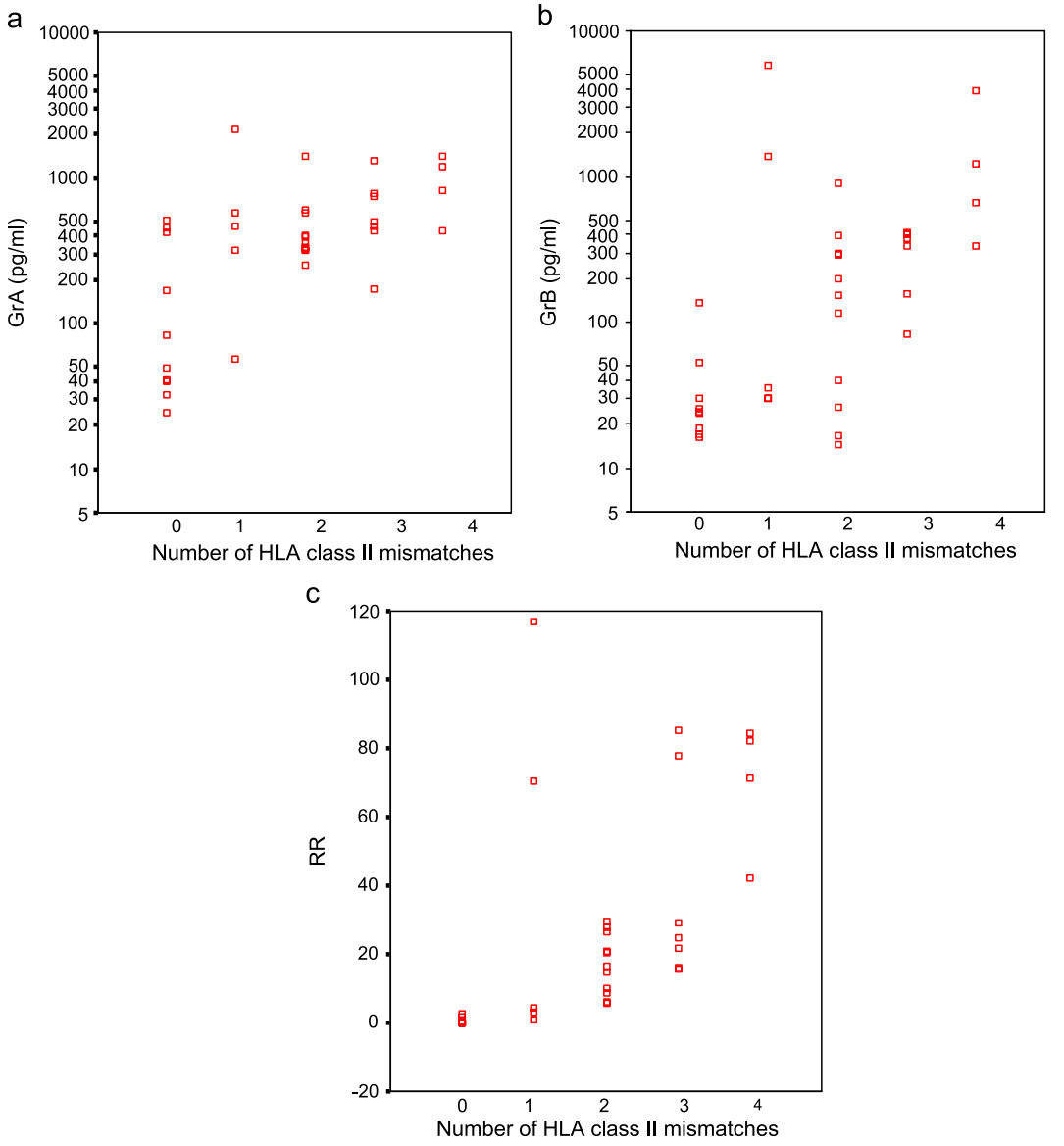

Fig. 2. Correlation of GrA, GrB production levels and RR of 37 patient/donor pairs with the number of HLA class II mismatches. On the x-axis, the number of HLA class II mismatches is shown. The y-axis represents the GrA (a) and GrB production levels (b) given on a logarithmical scale and the values of RR (c).

increases to 459 pg/ml (25–75% percentiles: 188–1366 pg/ml; p=0.0553 against no HLA class II mismatch) in the patient/donor pairs with one HLA class II mismatch (Fig. 2a). The GrA production levels of the patient/donor pairs with two HLA class II mismatches were not higher (median 357 pg/ml, 25–75% percentiles: 320–573 pg/ml; p=0.029 against no HLA class II mismatch) than the GrA production levels of the patient/donor pairs with one HLA class II mismatch. The GrA production levels of the patient/donor pairs with three HLA class II mismatches increased (median 496 pg/ml GrA, 25–75% percentiles: 435–786 pg/ml; p=0.0046 against no HLA class II mismatch), reaching the highest GrA production levels in patient/donor pairs with four HLA class II mismatches (median 1016 pg/ml GrA, 25–75% percentiles: 532–1369 pg/ml; p=0.008 against no HLA class II mismatch).

Similar results were obtained for GrB production levels. The GrB production levels of the patient/donor pairs without HLA class II mismatches were low (median 24 pg/ml GrB, 25–75% percentiles: 17–36 pg/ml; Fig. 2b); higher in the patient/donor pairs with one HLA class II mismatch (median 36 pg/ml GrB, 25–75% percentiles: 30–3579 pg/ml; p=0.028 against no HLA class II mismatch) and in the patient/donor pairs with two HLA class II mismatches (median 152 pg/ml GrB, 25–75% percentiles: 26–293 pg/ml; p=0.0242 against no HLA class II mismatch). In the patient/donor pairs with three HLA class II mismatches, a further rise of the median GrB production levels to 377 pg/ml (25–75% percentiles: 155–406 pg/ml; $p \leq 0.001$ against no HLA class II mismatch) was observed. Patient/donor pairs with four HLA class II mismatches showed the highest GrB production levels (median 949 pg/ml, 25–75% percentiles: 417–3220 pg/ml; p=0.002 against no HLA class II mismatch).

The median RR of the MLC in the patient/donor pairs without HLA class II mismatches was low (0.1%, 25–75% percentiles: 0.0–0.6%), significantly increased to 4.2% (25–75% percentiles: 2.0–94.0%; p=0.0027 against no HLA class II mismatch) in the patient/donor pairs with one HLA class II mismatch, to 16.5% (25–75% percentiles: 8.7–26.5%; $p \leq 0.001$ against no HLA class II mismatch) in the patient/donor pairs with two HLA class II mismatches and further increased to 24.7% (25–75% percentiles: 16.2–78.0%; $p \leq 0.001$ against no HLA class II mismatch) in the patient/donor pairs with three HLA class II mismatches (Fig. 2c). The highest RR was observed in patient/donor pairs with four HLA class II mismatches (77.0%, 25–75% percentiles: 49.4–84.0%; p=0.002 against no HLA class II mismatch).

3.3. Correlation of GrA and GrB production levels with the development of acute GvHD

In a separate, consecutive cohort consisting of 20 HLA-identical patient/donor pairs, GrA and GrB production levels were measured in the supernatants of pretransplant MLC established in graft-versus-host direction and correlated with the development of acute GvHD.

Fig. 3 shows the GrA production levels of seven patients without acute GvHD (grades 0–I), five patients with acute GvHD grade II, four patients with acute GvHD grade III and four patients with acute GvHD grade IV.

The median GrA levels in the supernatants of MLC of patients without acute GvHD (grades 0–I) was low (median 196 pg/ml GrA, 25–75% percentiles: 100–475 pg/ml) and significantly increased in

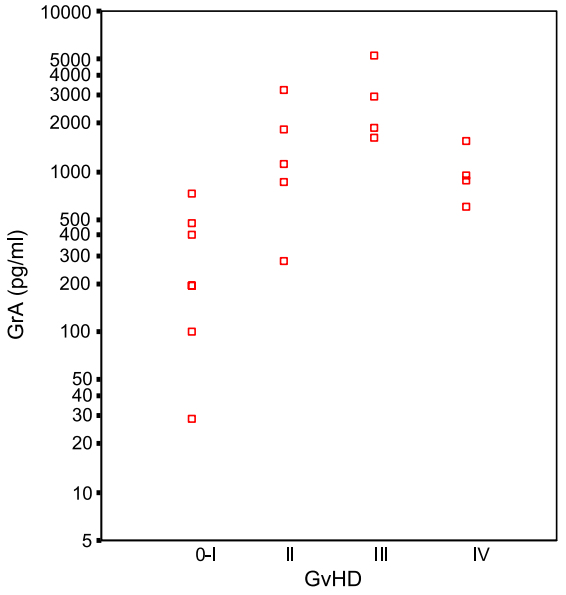

Fig. 3. Correlation of GrA production levels in the supernatants of MLC of 20 patient/donor pairs with the development of acute GvHD. On the x-axis, the different GvHD grades (0-I, II, III, IV) are shown. The y-axis represents the GrA production levels.

patients with acute GvHD grades II–IV (median 1533 pg/ml GrA, 25–75% percentiles: 869–2397 pg/ml; $p<0.001$; Fig. 3).

The median GrB production levels in the supernatant of MLC of 7 patients without acute GvHD (grades 0–I) was low (median 104 pg/ml GrB, 25–75% percentiles: 84–391 pg/ml) and increased, although not significantly, in the 13 patients who developed acute GvHD grades II–IV (median 207 pg/ml GrB; 25–75% percentiles: 97–375 pg/ml; $p=0.757$; data not shown).

4. Discussion

In the present study, GrA and GrB production levels were measured in the supernatants of pretransplant MLC and correlated with the RR of MLC, with the number of HLA class II mismatches and with the development of acute GvHD grades II–IV. The analysis was performed without prior knowledge of HLA typing, RR of the MLC and of development of acute GvHD.

We observed significant correlations between GrA and GrB production levels and RR of MLC ($r=0.492$ and $r=0.853$, respectively) and between GrA and GrB production levels and HLA class II mismatches ($r=0.579$ and $r=0.650$, respectively).

Significantly enhanced GrA and GrB production levels were already observed in the supernatants of MLC of one HLA class II-mismatched patient/donor pairs that further increased with the number of HLA class II mismatches. For example, the GrB production levels significantly increased from 24 pg/ml (median) in the zero HLA class II-mismatched patient/donor pairs to 36 pg/ml (median) in one HLA class II-mismatched patient/donor pairs and to 152 pg/ml (median) in two HLA class II-mismatched patient/donor pairs. We also analyzed whether mismatches for either HLA-DRB1, HLA-DQB1 or HLA-DPB1 would result in different GrA and GrB production levels. The three types of HLA class II mismatches all resulted in comparable enhanced levels of GrA and GrB production. HLA class I mismatches are likely to contribute to GrA and GrB production. Most of the patient/donor pairs with more than one HLA class II mismatch displayed similar numbers of HLA class I mismatches. The increase caused by the latter mismatches is expected to be comparable in our patient/donor combinations. In three HLA class II-matched patient/donor pairs with only HLA class I mismatches (HLA-B and HLA-C), low GrA and GrB production levels were measured. Yet, a thorough analysis on the additional impact of MHC class I mismatches on Gr production needs to be studied in a larger group of patient/donor pairs.

The pretransplant GrA production levels correlated with the development of acute GvHD grades II–IV after HLA-identical sibling SCT ($p<0.001$). Low GrA production levels were detectable in the supernatants of MLC of patient/donor pairs where the patient did not develop GvHD. The latter levels are comparable to those measured in the supernatant of cells with solely patients or donor cells (data not shown). Low Gr levels have also been observed in plasma of healthy individuals and reflect the constitutive release of proteases from activated Gr-producing lymphocytes (Spaeny-Dekking et al., 1998). Although the GrB levels showed increased levels in the patients with GvHD grades II–IV, the increment was not significantly different.

Analysis of the kinetics of the GrA and GrB production levels from day zero until day five showed peak levels at day four (data not shown). To exclude that the Gr production levels were not a reflection of different levels of cell death in the MLC, the number of $CD4^+$ and $CD8^+$ lymphocytes and dead cells were determined in a fluorescence-activated cell sorter. While $CD4^+$ T lymphocytes strongly increased, $CD8^+$ T lymphocytes and dead cells remained almost unchanged (data not shown). This suggests that the Gr production levels in the MLC can be mainly attributed to $CD4^+$ T lymphocytes which can also display cytolytic activity or to the small proportion of $CD8^+$ T lymphocytes and NK cells in MLC.

The patient groups (i.e., acute GvHD grades 0–I versus acute GvHD grades II–IV) were comparable in age and CMV status. The high GrA production levels, although measured in MLC of HLA-identical patient/donor pairs, suggest that mismatches in minor histocompatibility antigens can be detected by measurement of Gr as well. Patients with higher GrA (and GrB) levels developed GvHD grades II–IV. The significant association of GrA production levels with the development of acute GvHD in this first and small cohort of patients is striking; however, it needs to be confirmed in a larger cohort of patients and be

included in a multivariate analysis as additional risk factor for the development of acute GvHD.

The release of granzymes by CTLs and NK cells are key events in most cytotoxic reactions. Thus, GrA and GrB production reflects the immune effector functions relevant for GvHD. The role of both the perforin/granzyme and the Fas pathway in GvHD has mainly been investigated in murine GvHD models. In a MHC-mismatched model, bone marrow transplantation (BMT) with perforin-deficient T cells merely delayed onset of clinical GvHD and prolonged survival; yet, all mice ultimately died of severe acute GvHD. BMT of perforin-deficient cells in MHC-matched recipient mice not only delayed onset of GvHD symptoms but also reduced mortality (Levy et al., 1995). In a separate study analyzing the role of GrB in GvHD and rejection in GrB-deficient mice, a significant contribution of GrB in cytotoxicity, mediated by $CD8^+$ CTL but not by $CD4^+$ CTL, in acute GvHD was demonstrated (Graubert et al., 1996). It is likely that both perforin- and Fas-dependent cytolytic mechanism are important in the pathogenesis of GvHD. In recipients of MHC-disparate spleen cells lacking either perforin or functional FasL, clinical GvHD and death were delayed, but not prevented. In contrast, recipients of MHC-disparate spleen cells that lack both perforin and functional FasL did not develop GvHD and survived BMT (Braun et al., 1996).

In man, only indirect evidence for a potential role of the perforin/granzyme pathway in patients suffering from GvHD is available. Increased GrB, perforin and FasL mRNA transcripts were detected in patients with acute GvHD, although the levels did not correlate with clinical severity (Jaksch et al., 2003). In addition, some infiltrating T cells in early skin lesions expressed GrB and perforin mRNA (Clément et al., 1991b). Immunohistochemical studies indicated that perforin-positive T cells were present in skin biopsies of transfusion-associated GvHD and in some cases of acute and chronic GvHD (Sale et al., 1992; Takata, 1995; Higaki et al., 2001). Mouse models also suggest that the graft-versus-leukemia effect may be perforin-dependent (Tsukada et al., 1999; Schmaltz et al., 2001). In the patient cohort transplanted with HLA-identical siblings, no relapse was observed, irrespective of the levels of GrA and GrB production.

To date, next to the standard serological and molecular histocompatibility typing techniques, various cellular tests are used for functional matching (Lie et al., 2000). The latter are labor intensive and their usefulness is still debatable. Molecular-based typing is costly. In view of the still expanding HLA polymorphism combined with increasing numbers of unrelated SCT, not only a cheap, simple and quantitative but equally important, a functional donor selection procedure is really needed. Thus, a first screening procedure, among the various unrelated potential donors for allogeneic SCT by classical serological typing, followed by measurements of GrA and GrB production levels by a simple ELISA is an attractive proposition. As reactions of donor lymphocytes to recipient cells are not only dependent on mismatches of major HLA antigens but also on those of the minor histocompatibility systems, GrA and GrB production measurements may provide a tool for functional minor histocompatibility antigen matching among related donors as well. It is of interest to investigate whether the GrA and GrB ELISA can be used for solid organ donor selection for example in immunized renal patients.

Acknowledgment

This work was in part supported by the Erwin-Schrödinger Stipendium from the Austrian “Fonds zur Förderung der wissenschaftlichen Forschung” and “Verein zur Förderung Biologischer Therapien” (B.K.) and by an EC Grant BIOTECH No. BIO4-98-0236 to E.G. and A.M.D.

References

Beatty, P., Henslee-Downey, P.J., 2000. HLA mismatched family member hematopoietic stem cell transplantation. In: Atkinson, K. (Ed.), Clinical Bone Marrow and Blood Stem Cell Transplantation, 2nd ed. Cambridge Univ. Press, p. 16.

Braun, M.Y., Lowin, B., French, L., Acha-Orbea, H., Tschopp, J., 1996. Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal graft-versus-host disease. Blood 183, 657.

Clément, M.V., Haddad, P., Soulié, A., Benvenuti, C., Lichtenheld, M.G., Podack, E.R., Sigaux, N., Sasportes, M., 1991. Perforin and granzyme B as markers for acute rejection in heart transplantation. Int. Immunol. 3, 1175.

Clément, M.V., Soulié, A., Legros-Maida, S., Guillet, J., Gluckman, E., Sigaux, N., Sasportes, M., 1991. Perforin and granzyme B: predictive markers for acute GvHD or cardiac rejection after

bone marrow or heart transplantation. Nouv. Rev. Fr. Hematol. 33, 465.

Clément, M.V., Legros-Maida, S., Israel-Biet, D., Carnot, F., Soulié, A., Reynaud, P., Guillet, J., Gandjbakch, I., Sasportes, M., 1994. Perforin and granzyme B expression is associated with severe acute rejection. Transplantation 57, 322.

Deeg, H.J., Storb, R., 1984. Graft-versus-host disease: pathophysiological and clinical aspects. Annu. Rev. Med. 35, 11.

Fussel, S.T., Donnellan, M., Cooley, M.A., Farrell, C., 1994. Cytotoxic T lymphocyte precursor frequency does not correlate with either the incidence or severity of graft-versus-host disease after matched unrelated donor bone marrow transplantation. Transplantation 57, 673.

Graubert, T.A., Russell, J.H., Ley, T.J., 1996. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. Blood 87, 1232.

Griffiths, G.M., Namikawa, R., Mueller, C., Liu, C.-C., Young, J.D.-E., Billingham, M., Weissman, I., 1991. Granzyme A and perforin as markers for rejection in cardiac transplantation. Eur. J. Immunol. 21, 687.

Healey, G., Schwarer, A.P., 2002. The helper T lymphocyte precursor (HTLp) frequency does not predict outcome after HLA-identical sibling donor G–CSF-mobilised peripheral blood stem cell transplantation. Bone Marrow Transplant. 30, 341.

Higaki, Y., Yamada, O., Okamura, T., Mizoguchi, H., Kawashima, M., 2001. Granzyme-B-containing lymphocyte involvement in epidermal injury in graft-versus-host disease. Dermatology 202, 94.

Jaksch, M., Uzunel, M., Martinez Cangana, G., Remberger, M., Mattsson, J., 2003. Increased levels of immune transcript in patients with acute GvHD after allogeneic stem cell transplantation. Bone Marrow Transplant. 31, 183.

Kanda, Y., Tanaka, Y., Shirakawa, K., Yatomi, T., Nakamura, N., Kami, M., Saito, T., Izutsu, K., Asai, T., Yuji, K., Ogawa, S., Honda, H., Mitani, K., Chiba, S., Yazaki, Y., Hirai, H., 1998. Increased soluble Fas-ligand in sera of bone marrow transplant recipients with acute graft-versus-host disease. Bone Marrow Transplant. 22, 751.

Kuijf, M.L., Kwekkeboom, J., Kuijpers, M.A., Willems, M., Zondervan, P.E., Niesters, H.G., Hop, W.C., Hack, C.E., Paavonen, T., Hockerstedt, K., Tilanus, H.W., Lautenschlager, I., Metselaar, H.J., Kuijf, M.M., 2002. Granzyme expression in fine-needle aspirates from liver allografts is increased during acute rejection. Liver Transplant. 8, 952.

Levy, R.B., Baker, M., Podack, E.R., 1995. Perforin-deficient T cells can induce graft-versus-host disease after transplantation of MHC-matched or MHC disparate allogeneic bone marrow. Ann. N.Y. Acad. Sci. 770, 366.

Lie, J.L.W.T., Oudshoorn, M., Schreuder, G.M.T., Giphart, J.M.J., Claas, F.H.J., Van Rood, J.J., 2000. Histocompatibility typing procedures for selecting allogeneic hematopoietic stem cell donors. In: Atkinson, K. (Ed.), Clinical Bone Marrow and Blood Stem Cell Transplantation, 2nd ed. Cambridge Univ. Press, p. 1057.

Lieberman, J., 2003. Mechanisms of granule-mediated cytotoxicity. Curr. Opin. Immunol. 15, 513.

Liem, L.M., van Lopik, T., van Nieuwehuijze, A.E.M., van Houwelingen, H.C., Aarden, L., Goulmy, E., 1998. Soluble Fas levels in sera of bone marrow transplantation recipients are increased during acute graft-versus-host disease but not during infections. Blood 91, 1464.

Mickelson, E.M., Anasetti, C., Yoon Choo, S., Longton, G.M., Petersdorf, E.W., Martin, P.J., Hansen, J.A., 1993. Role of the mixed lymphocyte culture reaction in predicting acute graft-versus-host-disease after marrow transplants from haploidentical and unrelated donors. Transplant. Proc. 25, 1239.

Petersdorf, E.W., Gooley, T.A., Anasetti, C., Martin, P.J., Smith, A.G., Mickelson, E.M., Woolfrey, A.E., Hansen, J.A., 1998. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. Blood 92, 3515.

Russell, J.H., Ley, T.J., 2002. Lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol. 20, 323.

Sale, G.E., Podack, E., Browne, M., Myerson, D., 1992. Lymphocytes infiltrating lesions of human graft-versus-host disease (GvHD) contain perforin: direct evidence for cytolytic lymphocyte attack on epithelial cells in GvHD. Lab. Invest. 66, 108A.

Sasazuki, T., Juji, T., Morishima, Y., Kinukawa, N., Kashiwabara, H., Inoko, H., Yoshida, T., Kimura, A., Akaza, T., Kamikawaji, N., Kodera, Y., Takaku, F., 1998. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. New Engl. J. Med. 339, 1177.

Schmaltz, C., Alpdogan, O., Horndasch, K.J., Muriglan, S.J., Kappel, B.J., Teshima, T., Ferrara, J.L., Burakoff, S.J., van den Brink, M.R., 2001. Differential use of Fas ligand and perforin cytotoxic pathways by donor T cells in graft-versus-host disease and graft-versus-leukemia effect. Blood 97, 2886.

Schwarer, A.P., Jiang, Y.Z., Deacock, S., Brookes, P.A., Barrett, A.J., Goldman, J.M., Batchelor, J.R., Lechler, R.I., 1994. Comparison of helper and cytotoxic antirecipient T cell frequencies in unrelated bone marrow transplantation. Transplantation 58, 1198.

Spaeny-Dekking, E.H.A., Hanna, W.L., Wolbink, A.M., Wever, P.C., Kummer, A.J., Swaak, A.J.G., Middeldorp, J.M., Huisman, H.G., Froelich, C.J., Hack, C.E., 1998. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. J. Immunol. 160, 3610.

Takata, M., 1995. Immunohistochemical identification of perforin-positive cytotoxic lymphocytes in graft-versus-host disease. Am. J. Clin. Pathol. 103, 324.

Tiercy, J.-M., Roosnek, E., Mach, B., Jeannet, M., 1993. Bone marrow transplantation with unrelated donors: HLA class II oligonucleotide typing as predictive of mixed lymphocyte culture reactivity. Transplant. Proc. 25, 1243.

Tsukada, N., Kobata, T., Aizawa, Y., Yagita, H., Okumura, K., 1999. Graft-versus-leukemia effect and graft-versus-host disease can be differentiated by cytotoxic mechanisms in a murine model of allogeneic bone marrow transplantation. Blood 93, 2738.