Cell-Mediated Lysis of Human Hematopoietic Progenitor Cells

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Several techniques are available for the serological analysis of antigenic determinants on human hematopoietic progenitor cells (HPC). However, techniques for the recognition of cellularly defined antigens on such progenitor cells have not yet been described. We therefore developed an in vitro cellular cytotoxicity assay, with bone marrow cells as target cells. In this assay specific cytotoxic T lymphocyte (CTL) lines are used as effectors for cell-mediated cytolysis of bone marrow mononuclear cells that express the antigens for which the CTLs were primed in a mixed lymphocyte culture. As a model we used CTL lines against HLA-A2 or -B7 determinants. By using effector-target ratios varying from 1:2 to 4:1, 4 hr of incubation of these CTL lines with bone marrow mononuclear cells from HLA-A2 or -B7 positive donors resulted in a specific dose-dependent growth inhibition up to 100% of myeloid (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) HPC. In contrast, no inhibition of HPC was observed using mononuclear bone marrow cells from HLA-A2 or -B7 negative individuals as target cells. Experiments in which cell-cell contact was prevented showed that the antigen-specific lysis of HPC was dependent on intimate cell-cell contact between effector-CTLs and bone marrow target cells. Our results show that this cell-mediated cytotoxicity assay can be used as a sensitive and specific tool for the study of cellularly defined antigens on human hematopoietic progenitor cells.

INTRODUCTION

THE STUDY of polymorphic antigenic determinants on human HPC is relevant not only for the analysis of differentiation and regulation of hematopoiesis (1–7) but also for allogeneic bone marrow transplantation. In allogeneic bone marrow transplantation cell-mediated immunity against polymorphic determinants probably plays an important role in vivo. For instance, T lymphocytes of the recipient may recognize certain polymorphic antigenic determinants on HPC of the donor, leading to an immune-mediated rejection of the bone marrow graft (8–11). In particular, after removal of the immunocompetent T lymphocytes from the bone marrow graft to prevent acute graft-versus-host disease, an increase in graft rejections has been observed (11–15). Donor HPC may be killed in vivo in a cell-mediated lysis by residual, relatively radiation-resistant recipient T lymphocytes that are not killed by donor T lymphocytes (16). Therefore, since T lymphocyte depletion of the bone marrow graft is increasingly applied in allogeneic bone marrow transplantation, the mapping of polymorphic determinants on HPC is of major importance. The expression of antigenic determinants on HPC has been studied using serological techniques such as the complement-dependent cytotoxicity assay (2, 3, 17) and fluorescence-activated cell sorting (1, 3, 4, 18, 19)

However, until now, no in vitro assay has been available to investigate cellularly defined antigens on human HPC Such an assay is obligatory to detect polymorphic antigenic determinants that cannot be recognized serologically (20, 21)

In the present study we have developed a cell-mediated cytotoxicity assay for the detection of antigenic determinants on human hematopoietic progenitor cells. As a model we used CTL lines against several HLA class I antigens

MATERIALS AND METHODS

Establishment of Cytotoxic T Lymphocyte (CTL) Lines

CTL lines against HLA-A2 or -B7 determinants were established as previously described (22) Briefly, standard mixed lymphocyte cultures were established by incubating HLA-A2 or -B7 negative responder cells at a concentration of 5×10^5 cells/ml with HLA-A2 or -B7 positive irradiated (15 Gy) stimulator cells at a concentration of 5×10^5 cells/ml for 6 days in RPMI plus 15% serum (i e, HEPES-buffered RPMI 1640 with 15% prescreened pooled human AB serum supplemented with 0 1% gentamycin and 10 mM L-glutamine), at 37°C in an atmosphere of 5% CO_2 The effector T lymphocytes were harvested and further expanded for 3–5 days at a concentration of 10^5 cells/ml in a medium consisting of 20% T cell growth factor (Biotest, Offenbach, Germany) in RPMI plus 15% serum at 37°C in an atmosphere of 5% CO_2 The established CTL lines were then cryopreserved at a concentration of 10^7 cells/ml in a medium consisting of 70% RPMI, 20% human AB serum, and 10% dimethylsulfoxide and stored in liquid nitrogen Before use the CTL lines were thawed for 1 min in a 37°C water bath, diluted in RPMI plus 50% serum, washed once in the same medium, and further expanded for 3–5 days at a concentration of 10^5 cells/ml in 20% T cell growth factor in RPMI plus 15% serum at 37°C in an atmosphere of 5% CO_2 The cytotoxic reactivity and antigenic specificity of

Abbreviations *HPC, hematopoietic progenitor cells CTL, cytotoxic T lymphocyte, CFU-GM, colony forming unit-granulocytes macrophages, BFU-E, burst forming unit-erythrocytes, CFU-GEMM, colony forming unit-granulocytes, erythrocytes, macrophages, megakaryocytes, BMT, bone marrow transplantation FACS, fluorescence activated cell sorter, FBS, fetal bovine serum*

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the CTL lines were tested using a standard Cr^{51}-release assay (22) Surface marker analysis of CTL lines was performed using an indirect immunofluorescence technique with murine monoclonal antibodies and a fluorescence activated cell sorter (FACS analyzer, Becton-Dickinson Immunocytometry Systems, Mountain View, CA) (23) The expression of antigenic determinants on the effector cells was studied with monoclonal antibodies against the T cell markers CD3 (OKT3, Ortho Diagnostic Systems, Raritan, NJ), CD4 (Leu 3a, Becton-Dickinson Monoclonal Center Inc, Mountain View, CA), and CD8 (leu 2a, Becton-Dickinson), the B cell markers Leu 12 (Becton-Dickinson) and B1 (Coulter Clone, Coulter Immunology, Hialeah, FL), and HLA-DR (Becton-Dickinson), as well as using a monoclonal antibody recognizing the interleukin-2-receptor (TAC Becton-Dickinson)

Collection of Bone Marrow

Normal human bone marrow of donors for bone marrow transplantation was obtained, after informed concent, by aspiration from the posterior iliac crests The cells were collected in Hanks balanced salt solution with 100 units/ml of preservative-free heparin The marrow suspension was diluted in RPMI 1640 with 5% FBS (Gibco, Grand Island, NY) and centrifuged over Ficoll-Isopaque (1 077 g/cm^3, 1,000 *g*, 20 min, 20°C) The interphase cells were collected, washed twice in RPMI plus 5% FBS, and resuspended in the same medium For cryopreservation, bone marrow mononuclear cells at a concentration of 10^7 cells/ml were suspended in a medium consisting of 70% RPMI, 20% FBS, and 10% dimethylsulfoxide and frozen in a computer-controled freezer (Cryoson, Middenbeemster, The Netherlands) as previously described (2) Immediately before use the cells were thawed for 1 min in a 37°C waterbath, diluted in HEPES-buffered RPMI plus 20% FBS at 0°C, washed once in the same medium, and then washed again in RPMI plus 15% serum The cells were resuspended in RPMI plus 15% serum at a concentration of 5×10^5 viable cells/ml

Cell Mediated Cytotoxicity Assay

A quantity of $1\ 25 \times 10^5$ bone marrow cells in 0 25 ml RPMI plus 15% serum was mixed with an equal volume of this medium containing CTLs The effector-target cell ratios varied from 1 8 to 16 1 The cell mixture was centrifuged (1,000 *g*, 15 sec) to establish cell-cell contact between CTLs and bone marrow cells and then incubated for 4 hr in a fully humidified atmosphere of 5% CO_2 at 37°C After incubation, the cells were washed once in RPMI plus 15% serum and resuspended in α-modified Eagle's minimal essential medium (Flow Laboratories, Irvine, Scotland) with 20% FBS, and subsequently cultured for CFU-GM, BFU-E, and CFU-GEMM As a control to establish the necessity of cell-cell contact between CTLs and bone marrow cells, and to exclude the possibility of nonspecific inhibition of hematopoietic progenitor cell growth due to the presence of cytotoxic cells in the semisolid culture medium CTLs were added to bone marrow cells immediately before plating All CTLs were irradiated (20 Gy) before use to prevent colony formation by these cells

CFU GM

A quantity of 10^5 bone marrow cells was cultured in 1 ml medium containing 20% FBS (Rehatuin, Kaukakee, IL), 20% leukocyte-conditioned medium (24), 20% α-modified Eagle's minimal essential medium, and 40% methylcellulose 2 25% in a fully humidified atmosphere of 5% CO_2 at 37°C in 35-mm plastic dishes CFU-GM colonies defined as granulocytic, monocytic, or eosinophilic aggregates of more than 20 cells were scored on day 10 under an inverted microscope

BFU-E

A quantity of 10^5 bone marrow cells was cultured in 1 ml of medium containing 20% FBS (Rehatuin), 20% leukocyte-conditioned medium, 5% 10^{-3} M 2-mercaptoethanol, 5% Iscove's modified Dulbecco's medium, 5% deionized bovine serum albumin (Sigma, St Louis, MO), 5% human transferrin, and 40% methylcellulose 2 8% with 1 unit/ml erythropoietin (Connaught step III, Toronto, Canada) in 35-mm plastic dishes in a fully humidified atmosphere of 5% CO_2 at 37°C The number of BFU-E was scored on day 14

CFU-GEMM

A quantity of 10^5 bone marrow cells was cultured in 1 ml medium containing 30% ABO-compatible human heparin plasma, 7 5% phytohemagglutinin-leukocyte-conditioned medium (25), 5% 10^{-3} M 2-mercaptoethanol, 5% deionized bovine serum albumin, 5% human transferrin, 5% Iscove's modified Dulbecco's medium, and 40% methylcellulose 2 8% with 1 unit/ml erythropoietin (2 5%) in 35-mm plastic dishes in a fully humidified atmosphere of 5% CO_2 at 37°C CFU-GEMM, defined as colonies containing at least both erythroid and myeloid cells, was scored on day 14–18

Calculations

100% growth was defined as the number of colonies cultured from 10^5 untreated bone marrow mononuclear cells The number of colonies in these cultures was always within the normal range for our laboratory (CFU-GM 182 ± 15 (mean ± SE), BFU-E 121 ± 12, CFU-GEMM 16 ± 1) In cellular cytotoxicity assays the percentages of surviving HPC were calculated by dividing the total number of colonies by the number of colonies in the untreated control cultures

RESULTS

Characterization of CTL Lines

Table 1 shows the cytotoxic reactivity and antigenic specificity of the established CTL lines as tested against phytohemagglutinin-stimulated peripheral blood lymphocytes of HLA-A2 or HLA-B7 positive or negative donors In the Cr^{51}-release assay at an effector-target ratio of 10·1 the anti-A2-CTL line caused a $77 \pm 4\%$ lysis of A2-positive target cells and showed no reactivity ($8 \pm 1\%$ lysis) against A2-negative target cells The anti-B7-CTL line, using the same effector-to-target ratio, caused $92 \pm 7\%$ lysis of B7-positive target cells and $12 \pm 3\%$ lysis of B7-negative target cells As is shown in Table 1, surface marker analysis of the CTL lines showed that most of these cells were activated cytotoxic T lymphocytes

Cellular Cytotoxicity Assay on Bone Marrow Cells

Pilot studies using the anti-A2-CTL line (Fig 1) showed that incubation of these CTLs with A2-negative bone marrow cells did not influence plating efficiency of CFU-GM up to an effector-target ratio of 4.1 Control experiments using noncytotoxic PHA blasts showed similar results There was no inhibition of CFU-GM growth after incubation of PHA blasts with bone marrow cells up to a ratio of 8:1

When the anti-A2 CTLs were incubated with A2-positive bone marrow, inhibition of CFU-GM growth could be detected in a dose-dependent manner at effector-target

Table 1 Characterization of Cytotoxic T-Lymphocyte-Lines, Using a Cell-Mediated Lympholysis Assay Against PHA-Stimulated Peripheral Blood Lymphocytes

	Responder HLA Phenotype		Stimulator HLA Phenotype		% Lysis of Target Cells		Marker Analysis (% positive cells)
					A2 positive B7 negative	A2 negative B7 positive	
Anti A2 CTL line	A3	A11	A2		77 ± 4*	8 ± 1*	CD3 94 CD8 75 CD4 23
	B35	B51	B51				DR 88 TAC 18 B1 1
							Leu 12 2
Anti B7 CTL line	A1	A3	A1	A3	12 ± 3	92 ± 7	CD3 94 CD8 69 CD4 12
	B8	B35	B7	B8			DR 85 TAC 25 B1 1
							Leu 12 3

* Values are means ± SE of five experiments Effector target cell ratio 10 1

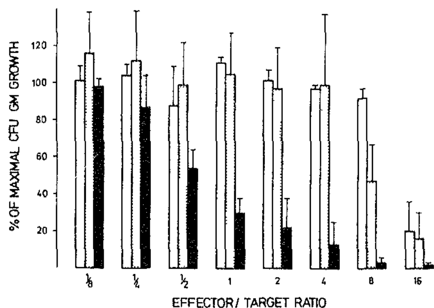

Figure 1 Growth of CFU GM after incubation of bone marrow cells with the anti A2-CTL line or noncytotoxic phytohemagglutinin blasts at various effector-target ratios Black bars, A2-positive bone marrow cells incubated with the anti-A2-CTL line (n = 3), stippled bars, A2-negative bone marrow cells incubated with the anti-A2-CTL line (n = 3), white bars, bone marrow cells incubated with phytohemagglutinin blasts (n = 3) Growth is expressed as a percentage of maximal growth in untreated control samples (means + SE)

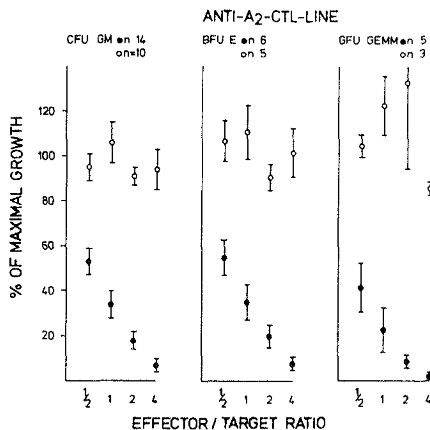

Figure 2 Growth of CFU-GM, BFU-E, and CFU-GEMM after incubation with the anti-A2-CTL line at various effector-target ratios Closed symbols, A2-positive bone marrow cells, open symbols, A2-negative bone marrow cells Growth is expressed as a percentage of maximal growth in the untreated control samples (means ± SE)

ratios from 1 2 upward Therefore, in further experiments effector-target ratios varying from 1 2 to 4 1 were used

As shown in Fig 2, by using effector-target ratios varying from 1 2 to 4 1 the anti-A2-CTL line effectively inhibited the growth of CFU-GM, BFU-E, and CFU-GEMM of A2-positive bone marrow donors but not of A2-negative individuals By using the same effector target ratios, incubation of the bone marrow cells with the anti-B7-CTL line (Fig 3) resulted in a significant inhibition of hematopoietic progenitor cell growth of B7-positive but not B7-negative individuals Adding the CTLs to the bone marrow cell culture at an effector-target ratio of 4 1 without incubation before plating did not result in inhibition of hematopoietic progenitor cell growth (Table 2)

DISCUSSION

Our results indicate that the cell-mediated cytotoxicity assay can be used to investigate the expression of cellularly defined antigenic determinants on hematopoietic progenitor cells

Using CTL lines against HLA-A2 or -B7 determinants, we demonstrated that these CTL lines effectively and specifically inhibited the growth of CFU-GM, BFU-E, and CFU-GEMM from A2- or B7-positive bone marrow donors, respectively, whereas no such inhibition was observed after treatment of bone marrow cells from A2- or B7-negative individuals Since relatively low effector-target ratios resulted in an almost complete specific inhibition of hematopoietic progenitor cell growth, this assay appears to be highly sensitive, although at the highest effector-target ratios some nonspecific inhibition occurred

This cell-mediated cytotoxicity assay may be particularly useful in studying the expression of minor transplantation antigens that cannot be recognized serologically (21, 21) These antigens probably play a major role in the etiology of graft-versus-host disease after allogeneic bone marrow transplantation and may conceivably play a role in graft failure which can occur after T lymphocyte depletion of the bone marrow graft (11–15) Although several pathophysiological mechanisms may be responsible for this graft failure, most cases are probably due to immunologic rejection of the transplant by the host (11, 13) This view is supported by the fact that the incidence of graft failure after T lymphocyte depletion of the bone marrow graft can be reduced by more intensive immunosuppressive

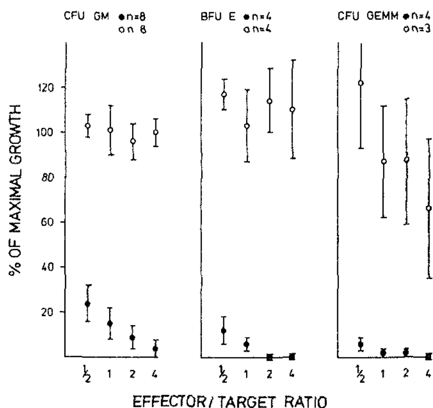

Figure 3 Growth of CFU-GM, BFU-E, and CFU-GEMM after incubation with the anti-B7-CTL line at various effector-target ratios Closed symbols, B7-positive bone marrow cells, open symbols, B7-negative bone marrow cells Growth is expressed as a percentage of maximal growth in the untreated control samples (means ± SE)

Table 2 Growth of Human Hematopoietic Progenitor Cells in the Presence of Antigen Specific CTLs with or without Incubation before Culture

	Incubation with CTLs	CTLs Added to Culture Medium	No of Experiments
CFU-GM	6 ± 2*	91 ± 6*	18
BFU-E	6 ± 2	86 ± 10	7
CFU-GEMM	1 ± 1	99 ± 21	6

* Values expressed as percentage of maximal colony growth in control cultures (means ± SE) Results using the anti A2 and anti B7-CTL lines were pooled Effector target cell ratio 4 1

regimens (11, 15, 26–28) Analysis of the nature of the effector cells involved, their interaction with bone marrow target cells, and the target determinants that are recognized in this process require the establishment of an in vitro model The technique described here may be very useful for this purpose Since the semisolid culture medium prevents cell-cell contract between the effector cells and the bone marrow target cells, it is possible, using this assay, to differentiate between processes that are dependent on cell-cell contact and other cell-dependent phenomena mediated by soluble factors Particularly in coculture studies investigating the influence of certain cell populations on growth of HPC, it may be important to distinquish between these processes (29) The assay opens the possibility of studying whether cell-mediated bone marrow graft rejection is restricted to polymorphic antigenic determinants or is due to nonspecific NK-cell-mediated lysis of HPC

In a murine model of graft rejection it was recently shown that the rejection process, as detected in vivo by proliferative spleen colony assays, could be studied in vitro using an assay similar to the technique described here (30) Incubation of bone marrow target cells with spleen-derived effector cells in vitro resulted in a specific growth inhibition of HPC, similar to the inhibition of HPC observed in vivo These data indicate that this in vitro assay is analogous to, and thus a useful model for, the in vivo situation It is to be expected that the cell-mediated cytotoxicity assay we have described here will be similarly useful in studying the mechanisms underlying cell-mediated bone marrow graft rejection in humans

We conclude that this cell-mediated cytotoxicity assay can be used as a sensitive and specific tool for the analysis of the expression of cellularly defined polymorphic antigens on hematopoietic progenitor cells and that this assay can be applied to study the mechanisms of cell-mediated graft rejections in allogeneic bone marrow transplantation

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