HLA Identical Leukemia Cells and T Cell Growth Factor Activate Cytotoxic T Cell Recognition of Minor Locus Histocompatibility Antigens In Vitro

PAUL M. SONDEL, JACQUELYN A. HANK, THAD WENDEL, BRIDGET FLYNN, and MAREK J. BOZDECH, *Departments of Pediatrics, Human Oncology, Genetics and Medicine of The University of Wisconsin, Madison, Wisconsin 53792*

ABSTRACT Lymphocytes from a healthy HLA-identical bone marrow transplant donor were tested for their ability to destroy her brother's acute myelogenous leukemia blasts in vitro. Primary mixed lymphocyte culture (MLC) and cell-mediated lysis (CML) responses between the patient's remission (pretransplant) and donor's lymphocytes were negative. Stimulation of donor lymphocytes for 7 d in vitro with irradiated leukemia cells, leukemia cells plus allogeneic irradiated lymphocytes, or a pool of irradiated lymphocytes from 10 donors, did not activate any cytotoxic cells able to destroy the HLA identical leukemic blasts. Further culturing for 7 additional d in T cell growth factor (TCGF) generated lymphocytes that induced effective cytotoxicity against the leukemic blasts, but not against autologous lymphocytes. Effective killing against the leukemia was observed only in cultures initially stimulated with the irradiated leukemia cells. These cytotoxic cells were maintained in TCGF and mediated persistent killing against the leukemic target cells. They were also able to destroy lymphocytes from the patient's mother and father, but not from an unrelated cell donor. This suggested specific recognition of non-HLA antigens inherited by the patient, that were foreign to the HLA identical bone marrow donor. These lymphocytes were cloned by a limiting dilution technique and one clone maintained cytotoxicity to the AML blasts and the father's lymphocytes, but not lymphocytes from the mother or an HLA-identical donor. This cytotoxicity was inhibited by a monoclonal anti-HLA antibody. Thus, in vitro sensitization of this sibling's lymphocytes with AML blasts followed by TCGF expansion, and cloning, enabled the detection of HLA-restricted cytotoxic cells that recognize minor locus histocompatibility antigens. This immune recognition may be relevant to the "graft vs. leukemia" effect that has been observed in leukemic animals and patients following histocompatible hematopoietic transplants.

INTRODUCTION

Human lymphocytes are readily activated in vitro by histocompatibility antigens controlled by the HLA region (1, 2). Lymphocytes from healthy donors who have never been directly immunized with foreign tissue mount a rapid proliferative response to foreign HLA-D antigens in the mixed lymphocyte culture (MLC)[1] and generate highly reactive cytotoxic T lymphocytes (T_c) that recognize antigens controlled by the HLA A, B, and C regions (3). In contrast, lymphocytes from healthy HLA-identical siblings do not induce either an MLC proliferative response nor the generation of cytotoxic cells that react to each others tissues in vitro (4). However, tissue grafts from one HLA identical sibling to another are rejected when immune suppression is omitted. Thus the "weak" minor histocompatibility antigens that can affect graft rejection in vivo are not recognized by human lymphocyte populations in a primary in vitro response (5).

In the mouse, primary responses to minor locus (non-H-2) antigens are observed between some strains (6). Priming in vivo increases the ability to detect a minor locus immune response in vitro (7). Recognition of

Dr. Sondel is a Scholar of the Leukemia Society of America and J. L. and G. A. Hartford Foundation Fellow. Dr. Hank is a Fellow of the Cancer Research Institute, New York. Dr. Bozdech is an American Cancer Society Junior Faculty Fellow. Address reprint requests to Dr. Sondel.

Received for publication 24 September 1982 and in revised form 15 February 1983.

[1] *Abbreviations used in this paper:* AML, acute myelocytic leukemia; BM, bone marrow; CML, cell-mediated lysis; GVD, graft vs. host; MLC, mixed lymphocyte culture; T_c, cytotoxic T lymphocytes; TCGF, T cell growth factor.

these minor locus antigens is H-2 restricted, since the T_c must simultaneously recognize H-2 antigens (8, 9). Furthermore, depletion of suppressor lymphocyte populations results in a population that gives an enhanced response to minor locus antigens in vitro (10). In man, in vivo immunization, either by multiple transfusions, or tissue grafting, has provided adequate sensitization to enable the detection of in vitro immune responses to minor locus transplantation antigens (11–13). These cytotoxic cells are also MHC restricted (14, 15).

We have studied the in vitro responses of human lymphocytes to HLA-identical leukemic myeloblasts. Prior reports have demonstrated that simultaneous in vitro sensitization with leukemic cells and unrelated histoincompatible cells can together act to induce the generation of cytotoxic lymphocytes able to destroy HLA-identical or autologous leukemic cells (16–18). We present here results of experiments investigating the in vitro response of a healthy lymphocyte donor to her brother's acute myelocytic leukemic (AML) cells. Initial in vitro sensitization with irradiated leukemia cells or allogeneic lymphocytes did not induce the generation of any antileukemic cytotoxicity. Subsequent expansion of these same in vitro primed lymphocytes in conditioned medium containing T cell growth factor yielded cytotoxic lymphocytes able to kill the leukemic blasts in vitro. Additional experiments were performed to determine the specificity of these cytotoxic lymphocytes. Assays using bulk or cloned cytotoxic T cells on leukemic and normal target cells demonstrated that reactivity was not against a "leukemia specific" antigen, but directed against minor locus histocompatibility antigens expressed on normal tissues.

METHODS

Lymphocytes and bone marrow cells. Lymphocytes were obtained by venipuncture from patients, their relatives, and healthy volunteers. Peripheral blood lymphocytes (PBL) were obtained by Ficoll sedimentation of heparinized blood. Heparinized bone marrow aspirates were obtained from leukemic patients and purified by Ficoll density flotation (16). All donors signed informed consent forms approved by the University of Wisconsin Committee for the Protection of Human Subjects.

Generation of conditioned medium containing T cell growth factor (TCGF). Pooled irradiated PBL from eight separate donors were cultured at 10^6 cells/ml in the presence of 1% phytohemagglutinin (Difco Laboratories, Detroit, MI). Supernatant was harvested after 48 h, filtered, and tested for potency by previously described methods (19, 20). Such unpurified supernatants are known to contain many lymphokines, some of which influence T cells, including interleukin-2 (19–22). This conditioned medium was used here to expand and clone human T cell populations; it is designated "TCGF" (19–25) to functionally distinguish it from the multiple other forms of conditioned medium that have been described.

In vitro cultures, MLC and cell-mediated lysis (CML) assays. MLC. The MLC was performed using conditions described previously (26). In brief, 0.1×10^6 responding cells were cultured in 0.25-ml round-bottomed Linbro microwells (Flow Laboratories, McLean, VA) with 0.1×10^6 irradiated stimulating lymphocytes (2,500 rad) or 0.2×10^6 irradiated (4,000 rad) cryopreserved AML blasts in 0.2 ml of HS-RPMI (media RPMI 1640 supplemented with 25 mM Hepes buffer, L-glutamine, penicillin, streptomycin [Flow Laboratories] and 15% [by volume] heat-inactivated nontransfused human male serum). After 6 d of incubation at 37°C in a 5% CO_2 humidified incubator, the cultures were pulsed with 1 μCi of ^{3}H-labeled methyl thymidine ([^{3}H]TdR) (New England Nuclear, Boston, MA). Radiolabeled cultures were incubated for 8 h and harvested with a MASH device (Otto Hiller, Madison, WI). The [^{3}H]TdR incorporation was measured in a liquid scintillation counter. Each experimental group was assayed in quadruplicate and expressed as mean counts per minute±the SEM.

CML. 10 million lymphocytes were cultured with various combinations of irradiated lymphocytes or AML blasts in 25-cm^2 flasks (Falcon Labware, Oxnard, CA) on end containing 15 ml of HS-RPMI. After 6 d in vitro, lymphocytes were harvested, resuspended, and serially diluted to give the appropriate effector to target (E:T) ratio as indicated in the text. Effectors were added in 0.1 ml to round-bottomed microwells. Target cells were fresh or cryopreserved lymphocytes or AML blasts cultured alone in HS-RPMI for 2–6 d immediately before use in the CML assay. Target cells were labeled with 250 μCi of ^{51}Cr (New England Nuclear) in 0.3 cm^3 for 3 h, then washed, diluted and 5×10^3 cells were added to the microwells in 0.1 ml. After addition of the target cells to the effector cells in the microwells, the plates were centrifuged at 200 *g* for 5 min at room temperature and then incubated for 4 h at 37°C. The plates were then centrifuged at 500 *g* at 4°C, supernatants harvested using Flow harvesting frames, and ^{51}Cr counted in a gamma counter. Percent cytotoxicity was calculated using the formula, percent cytotoxicity = (experimental cpm − spontaneous cpm/maximum cpm − spontaneous cpm) × 100. Spontaneous and maximum release were determined by incubating target cells in HS-RPMI or cetrimide detergent (Sigma Chemical Co., St. Louis, MO), respectively. Spontaneous release values ranged from 8 to 25% of the maximum counts per minute. In all experiments two or three effector to target ratios were tested.

Monoclonal antibody-CML inhibition. Hybridoma PA 2.6 derived by Dr. Peter Parham was obtained as a gift from Dr. Steven Shaw (National Institutes of Health, Bethesda, MD) and Dr. Peter Parham (Stanford University, Palo Alto, CA). This monoclonal reagent reacts with a monomorphic antigenic determinant thought to be present on all HLA class I (45,000 mol wt) antigens (27). This hybridoma was grown in ascites by Dr. William Sugden (University of Wisconsin). This ascites fluid was added to ^{51}Cr-labeled target cells at a final dilution of 1:200 just before addition of cytotoxic cells in experiments testing HLA restriction (28, 29). This same concentration of ascites fluid has been shown in our laboratory to inhibit specific lysis of allogeneic targets by bulk cultures of alloactivated T_c without inhibiting destruction of K562 target cells, and inhibit the HLA restricted recognition of some, but not all, T_c clones that destroy autologous lymphoblastoid cell line cells. We have also shown that other monoclonal antibodies have no inhibitory effect on cytolysis by these same T_c.[2]

Cryopreservation. Fresh lymphocytes, AML blasts, and in vitro activated lymphocytes were cryopreserved in 10%

[2] Hank, J. A., and P. M. Sondel. Submitted for publication.

dimethyl sulfoxide using controlled rate freezing (Cryo-Med, Mt. Clemens, MI) as described previously (16, 30).

RESULTS

A previously healthy 26-yr-old man (P) was diagnosed with acute myelogenous leukemia (by standard histologic criteria) in June 1981. Before any antileukemic therapy, an aliquot of his leukemic bone marrow (L) was aspirated, purified, and cryopreserved. Immunologic marker testing on these myeloblasts revealed they were typical AML cells; negative for terminal deoxynucleotidyl transferase, the "common" acute lymphoblastic leukemia antigen, and T and B cell markers, but heterogeneous for the Ia antigen (31). 4 mo later, while in a stable remission, he was given 1,000 rad fractionated total body irradiation, 60 mg/kg per d for 2 d of cyclophosphamide followed by 2.5×10^8/kg nucleated bone marrow cells from his HLA identical sister (S). HLA and MLC data are presented in Table I. Donor S is a healthy primiparous 45-yr-old white female, never transfused with allogeneic blood. Her only potential allosensitization was a full term pregnancy 13 yr before bone marrow (BM) donation.

Lymphocytes from S (obtained at the time of BM transplant donation) were cultured with irradiated leukemic cells (SL_x), with irradiated leukemic cells and irradiated lymphocytes from an unrelated donor (SL_xX_x), or with an irradiated pool of lymphocytes from 10 unrelated donors ($S\ Pool_x$). After 7 d of in vitro sensitization, these lymphocytes were tested for their cytotoxic capacity in the CML assay using leukemic blasts as target cells as well as other populations of target cells (Table II, experiment 1). Note that sensitization of the sister's lymphocytes with irradiated allogeneic cells (SX_x or $S\ Pool_x$) generated effective T_c directed against alloantigens present on target cells from unrelated donor X. However, neither of these cultures generated effective killing against the leukemic blasts (32). Even sensitization in the "three cell" protocol (SL_xX_x) generated no killing of the leukemic blasts (16, 17). However, this same culture was able to kill the allogeneic cells from donor X, demonstrating that immune activation had occurred in this culture. Lastly, lymphocytes from a second unrelated donor (Y) were sensitized with irradiated leukemic cells. This culture (YL_x) generated effective killing against the leukemic blasts, as well as against remission bone marrow cells from the patient, and lymphocytes from the healthy sibling. Thus, the leukemic blasts were effective at presenting alloantigens in a sensitization culture. Furthermore, the leukemic blasts, remission bone marrow, and sibling's lymphocytes could be destroyed by appropriately alloactivated killer cells recognizing the identical HLA antigens on these three target cells.

Replicate flasks of these same cultures were maintained in vitro in fresh media containing 20% TCGF. These were retested on the same target populations 7 d later (Table II, experiment 2). As noted in the primary cultures, alloactivation of sibling's (S) lymphocytes with irradiated pooled lymphocytes or cells from donor X induced T_c that effectively destroyed target lymphocytes from donor X. However, activation with the HLA identical leukemic blasts, alone (SL_x) or in combination with the irradiated allogenic cells (SL_xX_x), now induced 26.3 and 32.8% killing against the leukemic blasts. These same two effector cell populations also mediated weak killing against remission bone marrow (11.6% and 13.1% cytotoxicity), but were unable to destroy the sister's autologous lymphocytes.

Destruction of the leukemic cells and "remission" bone marrow by HLA identical sibling's lymphocytes could result from T_c recognition of minor locus (non-HLA) antigens present on both tissues, or from T_c de-

TABLE I
MLC Identity of Patient and Sister

Responding cells	[^{3}H]TdR cpm $\times 10^3$ induced by stimulating cells				
	Media	P_x	S_x	X_x	L_x
P	1.9±0.6	0.7±0.4	1.4±0.2	38.7±5.5	3.4±1.3
S	1.8±0.4	0.9±0.3	1.5±0.4	58.4±6.2	1.8±0.5
X	1.4±0.4	54.4±3.0	60.5±2.5	1.7±2.4	13.3±0.2

PBL (50×10^3 per well) from the patient (P), his HLA-identical sister (S), and an unrelated donor (X) were stimulated in MLC with irradiated (2,500 rad) lymphocytes (100×10^3) from donors P, S, or X, or the same number of irradiated (4,000 rad) cryopreserved AML blasts (L) from patient P. After 120 h, cultures were labeled with [^{3}H]TdR for 12 h and harvested. Results are [^{3}H]TdR cpm $\times 10^3$ of quadruplicate cultures±SEM. Both patient and donor were HLA A-2, A-X, B-15, B-17, and Bw4, Bw6 by standard serologic microcytotoxicity testing with alloantisera.

TABLE II

Generation of Cytotoxic Cells against HLA-identical AML Blasts

	Effector	Percent cytotoxicity on target cells			
		L	BM	S	X
Experiment 1					
Primary culture 7 d in vitro	SL_x	−2.0±0.4	−0.8±0.3	−1.2±0.4	−5.7±0.6
	SX_x	1.8±0.6	1.3±0.9	0.9±0.8	35.9±1.7
	S $Pool_x$	3.8±0.7	3.3±1.1	−1.6±0.4	24.9±9.5
	SL_xX_x	2.6±0.4	2.0±0.6	2.0±0.2	30.0±1.3
	YL_x	65.4±2.4	70.6±2.3	57.0±1.5	0.8±1.0
Experiment 2					
14 d in vitro (7 d in TCGF)	SL_x	26.3±0.8	11.6±1.7	−1.7±1.0	−0.5±0.4
	SX_x	0.3±0.5	0.9±1.5	0.7±0.4	55.7±1.5
	S $Pool_x$	7.3±0.5	1.7±1.0	0.1±0.6	56.2±1.7
	SL_xX_x	32.8±1.8	13.1±0.7	1.9±0.6	61.2±1.7
	YL_x	78.3±2.1	39.1±2.5	66.1±2.3	24.5±0.8

PBL (10×10^6) from the HLA-identical sibling (S) or an unrelated donor (Y) were stimulated in duplicate flasks with irradiated lymphocytes (10×10^6) from an unrelated donor (K_x), a pool of PBL from 10 unrelated donors ($Pool_x$) or AML cells (L_x) from patient P. After 7 d (experiment 1), cells from each sensitization mixture were harvested and tested in the cytotoxicity assay on the following target cells: L, AML blasts from P; BM, remission (pretransplant) nucleated bone marrow cells from P; S, PBL from donor S; X, PBL from donor X. For experiment 2, the duplicate flask of each sensitization combination was recultured after the addition of 20% (vol/vol) TCGF. After an additional 7 d in TCGF (day 14 of culture), lymphocytes were harvested and tested on ^{51}Cr-labeled target cells from the same four cell populations used in experiment 1. All results represent percent cytotoxicity±SD in a 4-h assay of 5×10^3 target cells and 150×10^3 effectors in quadruplicate wells.

tection of a "leukemia-specific" antigen present on both the AML blasts and the morphologically remission bone marrow. To differentiate these possibilities it was essential to test for cytotoxicity on other nonleukemic tissues that may be bearing the same minor locus antigens. Because this patient had already received an allogeneic bone marrow transplant, we were unable to recover any lymphoid populations from this patient that could clearly be of host origin and bear exactly the same minor locus antigens as the leukemic cells or remission bone marrow cells. We thus planned to test these effector cells on target lymphocytes from the parents of this patient. To prevent the loss of cytotoxic function of these effector cells while arranging for subsequent in vitro testing, these activated cytotoxic lymphocytes were cryopreserved.

PBL were obtained from both mother (M) and father (F) of this patient and also cryopreserved. After 6 mo in liquid nitrogen, the lymphocytes and effector cells were thawed and placed in culture and assayed in the chromium release test (Table III). As before, the sister's effector population that had been alloactivated (SX_x) was still highly efficient at destroying target cells from donor X. There was low level cross-reactive killing of this effector population on the mother's target cells and minimal cytotoxicity against the father's target cells. Minimal detectable cytotoxicity was seen against autologous lymphocytes from the sister or against the leukemic blasts by these alloactivated cytotoxic cells. In contrast, the leukemia cells were effectively destroyed by the sister's cells that had been activated with leukemic blasts (SL_x). This culture also mediated excellent killing against both mother's and father's target cells, but not against autologous lymphocytes (S) or cells from donor X.

To further examine the specificity of this T_c response, T_c clones were generated and maintained in vitro (23). Effector cells from the SL_x culture maintained in TCGF and tested in Table III were seeded at 1 cell/well into 192 microwells with 10^4 irradiated leukemic cells from the patient as feeder cells and 20% TCGF. After 2 wk, 23 clones showed abundant growth by visual inspection. These were tested in two separate screening assays and on each screening only clones 11 and 17 showed significant destruction of HLA-identical AML blasts (note that these clones have not been subcloned; even though they were initially seeded at one cell per well and recovered with a "cloning efficiency" of <23 per

TABLE III

Cytotoxic Cells Recognize Minor Histocompatibility Antigens

Cryopreserved effector	Stimulator	E:T	Percent cytotoxicity on target L	S	M	F	X
SL_x	L_x	10	24.3±1.3	3.7±1.4	25.8±2.8	34.9±2.9	0.3±0.7
		3	13.0±1.3	1.0±3.1	11.9±0.9	16.9±2.9	−2.0±0.5
SL_xX_x	X_x	10	6.2±0.5	−0.2±0.8	27.6±0.3	19.9±2.0	60.3±4.7
		3	3.0±0.8	−0.7±2.2	16.9±4.8	9.8±0.5	40.5±1.5
SX_x	X_x	10	3.5±0.5	3.5±2.2	16.8±1.5	5.7±0.6	47.5±1.3
		3	1.9±1.0	−0.1±1.2	7.9±1.3	6.7±0.8	32.3±0.7
YL_x	L_x	10	25.7±2.1	39.9±3.2	32.2±1.3	45.9±0.4	1.9±0.4
		3	12.0±1.3	22.5±0.8	9.3±0.8	23.1±1.7	−0.9±0.4

Excess effector cells harvested after 14 d in vitro (experiment 2, Table II) were cryopreserved in 10% dimethyl sulfoxide. 6 mo later these cells were thawed, cultured in TCGF, and restimulated with the original cryopreserved stimulating cells. Cultures were harvested after 7 d and tested on target cells: cryopreserved AML blasts from P (L), and chromated cryopreserved PBL from the patients HLA-identical sister (S), their mother (M), their father (F), and the unrelated donor (X). Cytotoxicity was measured in a 4-hr ^{51}Cr release assay with 5×10^3 target cells with both 15 and 50×10^3 effector cells in quadruplicate wells, making the effector to target (E:T) ratio, 10 and 3 to 1.

192 wells, it remains possible that they do not represent single T cell-derived clones). These clones were then tested (Table IV) for cytotoxicity against the same target cell populations tested previously with bulk generated T_c in Table III. In this test (and two subsequent tests) cells from clone 11 had lost their ability to destroy the AML blasts, as well as all other targets. In contrast, lymphocytes from clone 17 mediated significant destruction of the AML targets and the father's lymphocytes, but not target cells from the mother or autologous S targets. Table V shows that cytotoxicity on L and F targets by cells from clone 17 is inhibited by the PA 2.6 monoclonal antibody directed against a class I HLA monomorphic determinant. Thus, lymphocytes from clone 17 recognize a minor locus antigen on L and F target cells, and that recognition appears to be HLA restricted.

TABLE IV

Cloned Human T_c Recognize Minor Locus Antigen

Effector clone	Percent cytotoxicity on target L	S	M	F
Clone 11	5.6±2.5	−4.9±2.0	1.4±0.9	−1.9±2.5
Clone 17	26.3±3.3	6.2±1.1	2.2±2.2	15.5±3.6

Cells from T_c clones 11 and 17 (see text) were assayed on the indicated target cells in the 4-h ^{51}Cr release assay: L, AML blasts; S, M, and F are PBL from the patient's sister, mother, and father, respectively. Because of low effector cell yields, clone 11 was tested at 0.1 effector per target cell and clone 17 was tested at 1.0 effector per target cell.

DISCUSSION

These experiments demonstrate that under appropriate in vitro sensitization conditions, effector cells able to destroy HLA identical leukemic blasts can be generated and maintained in vitro, cryopreserved, thawed, recultured, cloned, and still retain their cytotoxic function and immunologic specificity. We feel that results presented in Table III demonstrate that non-HLA antigens are being recognized by the sister's lymphocytes that had been activated in vitro with irradiated leukemic cells and expanded with TCGF. The absence of killing by this population on autologous lymphocytes and target cells from donor X argues strongly against this being a totally "nonspecific" effector cell. In contrast, the activation of killing against the leukemic target might suggest the recognition of a leukemia specific antigen. But if this were the case, then this (SL_x) population should not have destroyed nonleukemic target lymphocytes from the mother and father. This latter effect could occur only if leukemia-specific antigens on the leukemic blasts are immunologically cross-reactive with foreign histocompatibility antigens on the mother's and father's normal lymphoid cells (this phenomenon has been designated "alien" histocompatibility antigens on tumor cells) (33). However, if this were the case, one would expect even greater killing of the unrelated X target cells by the SL_x culture since there is twice as great a chance of cross-reactivity on the target cells from unrelated donor X than on target cells from either the mother or father. This is due to donor X differing from the responding lym-

TABLE V

Killing by Cloned T Cells Directed against Minor Locus Antigen Is Inhibited by Monoclonal Anti-HLA Class I Antibody

Effector	E:T	Anti-HLA added	Percent cytotoxicity on target			
			L	S	M	F
Clone 17	12	−	30.1±1.2	2.0±0.5	2.0±1.0	28.8±1.7
	4	−	21.1±0.7	2.0±0.5	−1.2±1.1	12.7±1.1
	1	−	11.3±1.3	0.5±0.8	0.7±0.5	13.2±1.5
Clone 17	12	+	8.2±0.7	ND*	−0.8±0.5	−0.6±0.5
	4	+	4.5±0.7	ND	−0.1±0.8	0.9±1.6
	1	+	2.1±1.1	ND	2.0±0.5	6.9±5.1

Cells from clone 17 were tested on the indicated target cells at E:T ratios of 12, 4, and 1:1 in a 4-h ^{51}Cr release assay. Just prior to the addition of effector cells, PA 2.6 monoclonal antibody (1:200 final concentration of ascites fluid) was added to the target cells as indicated. Separate control (spontaneous and maximum release) values were obtained for target cells treated with PA 2.6 and these were not significantly altered from those values for nontreated target cells.

* ND, not determined.

phocyte donor by two HLA haplotypes rather than one haplotype, as is the case for both parents.

The activation of cytotoxic cells in the SL_x culture that can destroy L, M, and F targets, but not S or X targets (Table III) is most consistent with recognition of at least one minor locus transplantation antigen. There are numerous such loci able to activate murine allograft reactions in vivo; some loci induce stronger reactions than others (34). The responses demonstrated in Table III by the SL_x effector cells potentially reflect recognition of an allele controlled by a single non-HLA-locus. If so, the mother, father, and patient would all share an allele at that locus that was not inherited by the sister. Potential genotypes for this locus could be: father = *a/b*, mother = *a/b*, sister = *b/b*, and patient *a/b*. By this model the effectors from the SL_x culture in Table III would recognize the *a* allele as foreign on F, M, and L targets, but not on the S or X target cells. However, the clonal analysis (Tables IV and V) shows this single locus hypothesis to be an oversimplification. Recognition by cells from clone 17 of a determinant shared by the patient's leukemia cells and the father's lymphocytes, but not shared by the mother's lymphocytes, proves that killers from the bulk culture SL_x detected at least two separate minor locus determinants on L targets (Table III). While the determinant detected by clone 17 could potentially be a minor locus antigen controlled by the Y chromosome (14, 15), some other non-HLA antigen must be shared by M and L targets to account for killing of M targets (which contain no Y chromosome) by cells from the SL_x bulk culture (Table III). Thus, at least two alleles at one or more non-HLA loci are the targets of the killer cells stimulated by the leukemic blasts in these studies. In murine models, recognition of minor locus antigens by T_c is MHC restricted (7–9). That clone 17 effectors are inhibited by antibody to the class I HLA molecules is consistent with HLA-restricted recognition of this minor locus antigen, as demonstrated for human bulk cultures and T cell clones from multiply transfused donors (13–15).

Alloactivation with pooled lymphocytes, or a single allogeneic donor, can occasionally (but not always), activate antileukemic cytotoxicity (16, 32). The primary responses demonstrated in Table II show that the sister's lymphocytes are not activated to kill leukemic blasts by alloactivation alone. This was the case even after prolonged culturing in TCGF with repeat alloantigenic stimulation (SX_x, Table III) (35). In contrast, activation with leukemic cells and alloantigens together (SL_xX_x) did generate antileukemic killing in the 14-d culture. Thus in vitro activation of T_c able to destroy these HLA identical leukemic blasts required the presence of irradiated stimulating cells that expressed the same foreign minor locus antigens that were recognized on the leukemic cells in the CML assay. That this same SL_xX_x culture generated much less killing against the leukemic cells in Table III may be due to the fact that this culture was reactivated with TCGF, L_x, X_x after thawing, while the SL_x culture was reactivated only with TCGF and L_x. In other words, the presence of the alloantigenic stimulus (X_x) may have reactivated strong allorecognition in the bulk culture (SL_xX_x) that competitively inhibited the activation of "antileukemic" effectors recognizing minor locus antigens (36).

Previous studies demonstrating "primary" in vitro

cytotoxic responses to HLA-identical target cells bearing minor locus antigens all required extensive prior in vivo immunizations (4, 11–15). Thus, most responding cell donors were patients with aplastic anemia or renal failure receiving multiple transfusions or renal allografts. The healthy donor used here (S) was never transfused with any allogeneic blood product. It remains possible that her normal term pregnancy 13 yr before these studies caused in vivo immunization to fetal minor locus antigens. We were unable to obtain blood from her child or his father (unrelated to individuals S, P, M, or F) to screen for immune cross-reactivity between their minor locus antigens and those minor locus antigens of the AML patient (P) recognized as foreign by the donor. Nevertheless, no minor locus reactive T_c were detected following the primary in vitro stimulation of the sister's lymphocytes with irradiated AML blasts. These were detected only after further culturing in TCGF. This suggests that the primary culture caused the differentiation of cytotoxic precursors, but their detection required the "helper" or "expansion" signal provided by the TCGF (19, 21, 22, 37).

Preliminary evidence now suggests that immune responses across minor locus histocompatibility barriers can have an antineoplastic effect. In human bone marrow transplants using HLA-identical sibling donors, stronger graft vs. host (GVH) reactions (presumably a minor locus-directed immune response) are associated with a decreased chance of recurrent leukemia posttransplant (38, 39). The patient reported here had biopsy proven clinical stage I acute GVH of the skin on day 52 that spontaneously disappeared, and developed extensive chronic GVH on day 258 that responded well to cyclophosphamide and prednisone. He is well and in remission 16 mo posttransplant. In mouse, transplantation of H-2 matched, but minor locus mismatched, bone marrow can cure a strain AKR host of leukemia if given under appropriate conditions (40). These conditions seem to include alloimmunization of the donor before transplant with tissues bearing the same minor locus incompatibilities as the leukemic host (Shih, C. Y., R. L. Truitt, A. V. Lefever, L. D. Tempelis, M. M. Bortin, 1983, *J. Cell. Biochem.*, 7A: 78, (Abstr.), 41). Thus, the "graft vs. leukemia" response may actually represent preferential destruction of leukemic blasts by minor locus reactive lymphocytes.

Current in vitro methods now make it possible to grow adequate numbers of human lymphocytes for potential adoptive immunotherapy (33). Preliminary testing shows no significant toxicity in primates or human patients of T cells grown in TCGF (42, 43). The results reported here of minor locus reactive T_c stimulated by HLA identical AML blasts and expanded in TCGF, coupled with data regarding minor locus-induced graft vs. leukemia in mouse and man, suggest that minor locus-reactive human lymphocytes be tested for toxicity and antileukemic efficacy in clinical trials of human adoptive immunotherapy.

ACKNOWLEDGMENTS

We thank the staff of the University of Wisconsin Bone Marrow transplant team, and the family of P for their willing cooperation in these studies; Ms. Pam Reitnauer, Mr. Larry Brown, and Dr. Peter Kohler for their helpful suggestions and critical review of this manuscript; Ms. Debbie Minkoff and Ms. Gina Markson for technical assistance; and Ms. Mary Pankratz and Ms. Karen Blomstrom for manuscript preparation.

This research was supported by grants CH-237 of the American Cancer Society and R01-CA 32685-01 of the National Institutes of Health.

REFERENCES

1. Bach, F. H., M. L. Bach, and P. M. Sondel. 1976. Differential function of major histocompatibility antigens in T lymphocyte activation. *Nature (Lond.).* **259:** 273–281.
2. Benacerraf, B. 1981. Role of MHC gene products in immune regulation. *Science (Wash. DC).* **212:** 1229–1238.
3. Sondel, P. M., and F. H. Bach. 1975. Recognitive specificity of human cytotoxic T lymphocytes. I. Antigen-specific inhibition of human cell-mediated lympholysis. *J. Exp. Med.* **142:** 1339–1348.
4. Sondel, P. M., and F. H. Bach. 1976. Recognitive specificity of human cytotoxic T lymphocytes. II. The nonrecognition of antigens controlled outside the major histocompatibility complex. *Tissue Ant.* **7:** 173–180.
5. Ceppellini, R., E. S. Curtoni, G. Leigheb, P. L. Mattiuz, V. C. Miggiano, and M. Visetti. 1965. An experimental approach to the genetic analysis of histocompatibility in man. *Histocompatibility Testing.* Munksgaard, Copenhagen. 13–22.
6. Macphail, S., I. Yron, and O. Stutman. 1982. Primary in vitro cytotoxic T cell response to non-MHC alloantigens in normal mice. *J. Exp. Med.* **156:** 610–621.
7. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* **142:** 1349–1364.
8. Hunig, T. R., and M. J. Bevan. 1982. Antigen recognition by cloned cytotoxic T lymphocytes follows rules predicted by the altered-self hypothesis. *J. Exp. Med.* **155:** 111–125.
9. Roopenian, D. C., M. B. Widmer, C. G. Orosz, and F. H. Bach. 1983. Helper cell-independent cytolytic T lymphocytes specific for a minor histocompatibility antigen. *J. Immunol.* **130:** 542–545.
10. Hayes, C. E., S. Macphail, and F. H. Bach. 1980. Generation of primary cytotoxic lymphocytes against nonmajor histocompatibility complex antigens by anti-Ia serum plus complement treated lymphocytes. *J. Exp. Med.* **151:** 1305–1310.
11. Garovoy, M. R., V. Franco, D. Zschaeck, C. B. Carpenter, T. B. Strom, and J. P. Merrill. 1973. Direct lymphocyte-mediated cytotoxicity as an assay of presensitization. *Lancet.* **I:** 573–576.

12. Yust, I., J. Wunderlich, D. L. Mann, G. N. Rogentine, B. Leventhal, R. Yankee, and R. Graw. 1974. Human lymphocyte dependent antibody mediated and direct lymphocyte cytotoxicity against non-HLA antigens. *Nature (Lond.).* **294:** 263–265.
13. Zier, K., W. Elkins, and G. R. Pierson. 1982. Cytotoxic T cell lines (CTLL) against a human minor alloantigen. *Immunogenetics.* **15:** 501–508.
14. Goulmy, E., A. Termijtecen, B. A. Bradley, and J. J. Van Rood. 1977. Y antigen killing by women is restricted by HLA. *Nature (Lond.).* **266:** 544–545.
15. Goulmy, E., A. Van Leeuwen, E. Blockland, J. J. Van Rood, and W. E. Biddeson. 1982. Major histocompatibility complex restricted H-Y specific antibodies, and cytotoxic T lymphocytes may recognize different self determinants. *J. Exp. Med.* **155:** 1567–1572.
16. Sondel, P. M., C. O'Brien, L. Porter, S. F. Schlossman, and L. Chess. 1976. Cell-mediated destruction of human leukemic cells by MHC identical lymphocytes: requirement for a proliferative trigger *in vitro*. *J. Immunol.* **117:** 2197–2203.
17. Zarling, J. M., M. McKeough, P. Raich, and F. H. Bach. 1976. Generation of cytotoxic lymphocytes *in vitro* against autologous human leukemia cells. *Nature (Lond.).* **262:** 691–693.
18. Lee, S. K., and R. T. D. Oliver. 1978. Autologous leukemia specific T cell mediated lymphocytotoxicity in patients with AML. *J. Exp. Med.* **147:** 912–922.
19. Smith, K. A., L. B. Lackman, J. J. Oppenheim, and M. F. Favata. 1980. The functional relationship of the interleukins. *J. Exp. Med.* **151:** 1551–1556.
20. Hank, J. A., H. Inouye, L. A. Guy, B. J. Alter, and F. H. Bach. 1980. Long term maintenance of "cloned" human PLT cells in TCGF with LCL cells as a feeder layer. *J. Supramol. Struct.* **13:** 525–532.
21. Finke, J. H., J. Scott, S. Gillis, and M. L. Hilfiker. 1983. Generation of alloreactive cytotoxic T lymphocytes: evidence for a differentiation factor distinct from IL 2. *J. Immunol.* **130:** 763–767.
22. Garman, R. D., and D. P. Fan. 1983. Characterization of helper factors distinct from interleukin 2 necessary for the generation of allospecific cytolytic T lymphocytes. *J. Immunol.* **130:** 756–762.
23. Lotze, M. T., J. L. Strausser, and S. A. Rosenberg. 1980. *In vitro* growth of cytotoxic human lymphocytes. II. Use of T cell growth factor (TCGF) to clone human T cells. *J. Immunol.* **124:** 2972–2978.
24. Morgan, D. A., F. W. Ruscetti, and R. C. Gallo. 1976. Selective growth of T-lymphocytes from normal human bone marrows. *Science (Wash. DC).* **193:** 1007.
25. Inouye, H., J. A. Hank, B. Alter, and F. H. Bach. 1980. T cell growth factor (TCGF) production for cloning and growth of functional human T lymphocytes. *Scand. J. Immunol.* **12:** 149–154.
26. Hank, J. A., and P. M. Sondel. 1982. Soluble bacterial antigen induces specific helper and cytotoxic responses by human lymphocytes in vitro. *J. Immunol.* **128:** 2734–2738.
27. Brodsky, F. M., and P. Parham. 1982. Monomorphic anti HLA A, B, C monoclonal antibodies detecting molecular subunits and combinational determinants. *J. Immunol.* **128:** 129–135.
28. Weiss, A., H. R. MacDonald, J. C. Cerottini, and K. T. Brunner. 1981. Inhibition of cytolytic T lymphocyte clones reactive with moloney leukemia virus-associated antigens by monoclonal antibodies: A direct approach to the study of H-2 restriction. *J. Immunol.* **126:** 482–485.
29. McMichael, A. J., P. Parham, F. M. Brodsky, and J. R. Pilch. 1980. Influenza virus specific cytotoxic T lymphocytes recognize HLA molecules: blocking by monoclonal anti-HLA antibodies. *J. Exp. Med.* **152:** 195s–203s.
30. Chess, L., G. N. Bock, and M. R. Mardiney. 1972. Restoration of the reactivity of frozen stored human lymphocytes in the mixed lymphocyte reaction and in response to specific antigens. *Cryobiology.* **14:** 728–733.
31. Sondel, P. M., W. Borcherding, N. T. Shahidi, D. J. Ganick, J. C. Schultz, and R. Hong. 1981. Recategorizing childhood ALL with monoclonal antibodies to human T cells. *Blood.* **57:** 1135–1137.
32. Zarling, J. M., H. I. Robins, P. C. Raich, F. H. Bach, and M. L. Bach. 1978. Generation of cytotoxic T lymphocytes to autologous human leukemia cells by sensitization to pooled allogeneic normal cells. *Nature (Lond.).* **274:** 269–271.
33. Sondel, P. M., and J. A. Hank. 1981. Alien drive diversity and alien selected escape: a rationale for allogeneic cancer immunotherapy. *Transplant. Proc.* **XIII:** 1915–1921.
34. Halle-Pannenko, O., L. L. Pritchard, and G. Mathe. 1981. Minor histocompatibility antigens and lethal graft versus host reaction in adult mice. *In* Graft versus Leukemia in Man and Animal Models. J. P. Okunewick and R. F. Meredith, editors. C.R.C. Press, Boca Raton, FL. 192–207.
35. Zarling, J. M., and F. H. Bach. 1980. Enhanced cytotoxicity of allosensitized T cells for autologous lymphoblastoid cell lines and human leukemia cells following propagation in T-cell growth factor. *Transplant. Proc.* **XII:** 164–166.
36. Sondel, P. M., M. W. Jacobson, and F. H. Bach. 1975. Pre-emption of human cell mediated lympholysis by a suppressive mechanism activated in mixed lymphocyte cultures. *J. Exp. Med.* **142:** 1606–1611.
37. Cheever, M. A., P. D. Greenberg, A. Fefer, and S. Gillis. 1982. Augmentation of the anti-tumor therapeutic efficacy of long term cultured T lymphocytes by in vivo administration of purified interleukin-2. *J. Exp. Med.* **155:** 968–980.
38. Weiden, P. L., N. Flournoy, E. D. Thomas, R. Prentice, A. Fefer, C. D. Buckner, and R. Storb. 1979. Antileukemic effect of allogeneic-marrow grafts. *N. Engl. J. Med.* **300:** 1068–1073.
39. Weiden, P. I., K. M. Sullivan, N. Flournoy, N. R. Storb, and E. D. Thomas. 1981. Antileukemic effect of chronic G.V.H. disease. Contribution to improved survival after allogeneic marrow transplantation. *N. Engl. J. Med.* **304:** 1529–1532.
40. Bortin, M. M., R. L. Truitt, and A. Rimm. 1979. Graft versus leukemia reactivity induced by alloimmunization without augmentation of graft versus host reactivity *Nature (Lond.).* **281:** 490–491.
41. Truitt, R. L., C-Y. Shih, A. A. Rimm, L. D. Tempelis, and M. M. Bortin. 1981. Alloimmunization and adoptive immunotherapy of leukemia. *Transplant. Proc.* **13:** 1910–1914.
42. Slease, R. B., D. M. Strong, K. E. Gawith, and G. D. Bonnard. 1981. Clinical effects of infusions into chimpanzees of primed autologous cultured T cells. *J. Natl. Cancer Inst.* **67:** 489–493.
43. Lotze, M. T., B. R. Line, D. J. Mathisen, and S. A. Rosenberg. 1980. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): implications for the adoptive immunotherapy of tumors. *J. Immunol.* **125:** 1487–1493.