Cancer Immunol Immunother (1993) 37: 187–194

Cancer Immunology Immunotherapy

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Detection of melanoma-reactive CD4+ HLA-class I-restricted cytotoxic T cell clones with long-term assay and pretreatment of targets with interferon-γ

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Received: 20 October 1992 / Accepted: 5 February 1993

Abstract. Twenty-five CD4+ cytotoxic T lymphocyte (CTL) clones were obtained from the peripheral blood or tumor tissues of melanoma patients undergoing active specific immunotherapy. Melanoma-reactive T cells were cloned by limiting dilution using either autologous or allogeneic melanoma cells to stimulate their proliferation. Sixteen of the clones reacted against autologous melanoma cells but not against the autologous lymphoblastoid cell line, which we defined as "melanoma-specific." Optimal demonstration of the lytic activity of CD4+ CTL required a 16-h incubation period and an effector:target cell ratio of 40:1. In addition, a 24-h pre-incubation of the target melanoma cells with 100 U interferon (IFN)γ consistently augmented lysis by these CD4+ CTL, increasing it from a mean level of 20% to one of 52%. Lysis by 8 of the 11 melanoma-reactive CD4+ T cell clones was exclusively HLA-class-I-restricted, as judged by blocking with monoclonal antibodies (mAb). Five of these HLA class-I-restricted clones were reactive only with the autologous melanoma cells, while the other 3 clones were also reactive with allogeneic melanoma cells. In all cases, the T cells and melanoma targets shared at least one HLA class I allele, usually HLA-A2, HLA-C3 or HLA-B62. Interestingly, lysis by 2 of the 11 clones was inhibited by both anti-HLA-class-I or -HLA-class-II mAb, while lysis by 1 other clone was inhibited by neither. HLA class I molecules and several accessory molecules were maximally expressed by the melanoma target cells, both in terms of distribution and copy number before IFNγ treatment. Thus, IFNγ may have acted by increasing the expression of melanoma-associated epitopes as presented by HLA class I (or HLA class II) molecules. A proportion of human CD4+ CTL appeared to recognize melanoma-associated epitopes presented by the HLA class I molecule, although their lytic potency may be less than that of their CD8+ counterparts.

Key words: Lymphocytes – Cytokines – Antitumor – Cell-mediated immunity – ^{51}Cr-release

Introduction

Human melanoma was for many years presumed to be an immunologically responsive tumor because spontaneous regressions, though rare, had been observed. More recently, clinical trials with active specific immunotherapy [10, 19–22] or interleukin-2 and adoptive transfer of tumor-infiltrating lymphocytes [28–30] have provided more substantive evidence for the effectiveness of immunological treatments for metastatic melanoma, albeit in a minority of patients.

It has become generally accepted that the T lymphocyte is central to an effective antitumor response in melanoma as well as other malignant diseases [19, 20, 27]. In the human peripheral blood, CD4 and CD8 molecules are expressed by two mutually exclusive subsets of T cells. CD molecules have been thought to correlate with T cell function and their ability to interact with major histocompatibility complex (MHC) products. In the human antitumor response, as in the mouse, specific killing of melanoma cells has usually been ascribed to the CD8+ T cell subset, which exhibit specificity for autologous and/or allogeneic melanoma cells [15, 16, 27]. However, it has been shown in our laboratory, as well as by others, that specific lysis of melanoma cells can also be mediated by CD4+ T cells [9, 23, 24]. In fact, under our conditions of cell culture, with interleukin-2 (IL-2) alone as a growth factor, approximately equal numbers of cytotoxic CD4+ and CD8+ were generated in many mixed lymphocyte/tumor cell cultures. CD4+ T cells were invariably less efficient at killing their target cells than were CD8+ cells [15]. Since melanoma

This work was supported by USPHS grant R01-CA 36233, and a grant from the Concern Foundation for Cancer Research.

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cells lacking MHC class II antigens were also lysed by some CD4+ T cell clones, we reasoned that recognition at least in those instances was not mediated by the MHC class II molecule [9]. This was clearly at odds with studies of hapten- or virus-specific cytotoxic CD4+ T lymphocytes (CTL) [4, 7, 12, 17], where CD4+ T-cell-mediated cytolysis was invariably MHC-class-II-restricted. The percentage of melanoma cells in situ that express HLA class I far exceeds that expressing HLA class II molecules [25] (Mitchell MS et al., in preparation). Also, tumor-associated epitopes are endogenous peptides. Both of those conditions would require that effective cytolytic T cells preferentially recognize melanoma epitopes in an HLA class I context.

In this paper, we have re-examined the potency of CD4+ CTL and have attempted to develop assay conditions to optimize their detection. We have examined the effect of pretreating the melanoma target cells with IFNγ on their susceptibility to lysis and extended the incubation period to include long-term killing. Monoclonal antibodies (mAb) to HLA class I or HLA class II molecules were then used to determine the MHC restriction of the cloned cytotoxic CD4+ T cells that we identified.

Materials and methods

Establishment of human melanoma cell cultures and other cell lines. The melanoma cell lines MSM-M-1 (abbreviated M-1), MSM-M-2 (M-2) and MSM-M-14 (M-14) were generated in our laboratory from biopsies of subcutaneous metastatic lesions from various patients [19–21]. Melanoma cells M-14 were obtained from a biopsy from patient WB, and so were autologous with the WB CTL in our experiments. The cell lines were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). All of the melanoma cell lines were adherent to plastic and were removed from the culture flasks by incubation with 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline.

A lymphoblastoid cell line, LCL-14, was established from peripheral blood lymphocytes of the patient WB by Epstein-Barr virus transformation, with a virus-containing supernate from the B95-8 cell line (Courtesy of Dr. Marshall R. Posner, New England Deaconess Hospital, Boston, Mass.). Unfortunately, despite several attempts, we were unable to produce a lymphoblastoid cell line (LCL) corresponding with melanoma M-1.

All cultures were tested for *Mycoplasma* contamination every 6 weeks by Hoechst staining for extranuclear DNA. None contained *Mycoplasma*.

Isolation of CD4+ T cell clones. Peripheral blood lymphocytes (PBL) or tumor-infiltrating lymphocytes (TIL) were isolated from four melanoma patients (CC, GW, RB and WB) undergoing active specific immunotherapy. All had received at least five injections of a therapeutic melanoma vaccine ("melanoma theraccine") consisting of disrupted M-1 and M-2 melanoma cells and the adjuvant Detox [19–21]. PBL and TIL were labeled with fluorescein isothiocyanate(FITC)- or phycoerythrin(PE)-conjugated anti-leu3a(CD4) mAb or anti-leu2a (CD8) mAb (Becton Dickinson, Mountain View, Calif.). The mutually exclusive subsets CD4+ CD8− or CD4− CD8+ were sorted with a fluorescence-activated cell sorter (FACS, Becton Dickinson). Sorted CD4+ CD8− cells were placed into 96-well round-bottomed microtiter plates at concentrations of 1, 10, or 100 cells per well, and were cultured with the following: (a) 5000/well irradiated (2500 Gy) autologous melanoma cells M-14 (for patient WB) or the allogeneic melanoma cells M-1 (for patients GW, CC and RB), which is one of the components of the melanoma theraccine, (b) 5000 cells/well irradiated (2500 Gy) autologous PBL or the LCL-14 used as a source of antigen-presenting cells, and (c) 100 Cetus units (600 IU)/ml IL-2. The cells were cultured in HEPES-buffered RPMI-1640 medium containing 10% human AB serum (Gemini Bio-Products Inc., Calabasas, Calif.), 2 mM L-glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Sigma Chemical Corp. St. Louis, Mo.). After 7–12 days, growth medium was replaced with fresh medium containing 100 Cetus units/ml IL-2.

Approximately 3 weeks later, CD4+ T cell clones were isolated from the 96-well plates that contained fewer than 20 positive wells. The putative clones were further expanded in the presence of either M-14 or M-1 irradiated melanoma cells, irradiated antigen-presenting cells, 100 U/ml IL-2 and 150 U/ml interleukin-4 (IL-4). Subsequently, the cultures were restimulated every week with irradiated melanoma cells and antigen-presenting cells in the same medium. Each T cell clone was retested for its CD phenotype and was confirmed to be CD4+ CD8−. Cytotoxicity was determined approximately 2 months after the limiting dilution.

Although putative CD4+ T cell clones in this study were generated by limiting dilution, we recognized the possibility that some of them were not pure clones. Under our conditions of culture, it was impossible to subclone and retain viability. A confirmation of clonality would be strongly supported by finding a specific single sequence of the T cell receptor (TCR) gene. Owing to the limited number of T cells we could generate from each clone, we were unable to perform the sequencing of the gene encoding the TCR on all clones. However, of the four putative clones for which the TCR was sequenced by the polymerase-chain reaction (WB25, 83, 84, and 91), all had only one pair of Vα and Vβ gene sequences. Moreover, each had only one D-J-C region. Thus, the weight of evidence presently supports the suggestion that a single clone was derived from each of these single wells. These studies on the TCR were performed as part of a larger collaboration by Dr. Elwyn Y. Loh, Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, Pa.

HLA phenotyping was kindly performed by Dr. Paul I. Terasaki at the University of California at Los Angeles. The HLA phenotypes of the patients and melanoma cell lines are summarized in Table 1. There was some difficulty in serotyping the melanoma cell lines, which led to an apparent discrepancy in the phenotypes of M-1 melanoma and frozen peripheral blood lymphocytes (not LCL) from the donor of the line. It is likely that the lymphocyte phenotypes were more accurate. Note that HLA-A2 and HLA-A28 are also serologically cross-reactive molecules, and that HLA-B12 and HLA-B44 are members of the HLA-B12 "split."

Table 1. HLA types of patients and melanoma cell lines

Cell source	Class I antigens			Class II antigens	
	A	B	C	DR	DQ
Patients					
CC	2,3	7,60	3	4,14	5,8
GW	1,3	7,8	7	1,3	2,5
RB	1,2	8,27	2,7	17	2
WB	2,29	44,62	3	7,13	6,7
Melanoma cell lines					
M-1	28 (2)[a]	60 (12,62)	3,6	4,10	8
M-2	28	51,60	6		
M-14	2,29	44,62	3		

[a] HLA type determined from patient's lymphocytes is in parentheses

Determination of cytotoxicity by ^{51}Cr-release assay. The cytotoxicity of CD4+ T cell clones against melanoma cell lines was tested by a ^{51}Cr-release assay [9, 15, 16]. A sample containing 2500 ^{51}Cr-labeled target cells was plated into each well of 96-well round-bottomed microculture plates in a final volume of 200 µl. Effector cells (cloned CD4+ T cells) were added in triplicate at effector:target (E:T) ratios of 20:1 or 40:1. The plates were centrifuged at 1000 rpm for 5 min and then incubated for either 6 h or 16 h at 37°C. To estimate the amount of released ^{51}Cr, 50 µl supernatant fluid was harvested and counted in an automated 5500 Beck-

man gamma counter (Beckman Instruments, Irvine, Calif.). The percentage cytotoxicity was calculated as $100 \times$ [soluble ^{51}Cr (cpm) in test wells – spontaneous release]/[total ^{51}Cr (cpm) – spontaneous release]. In 16-h ^{51}Cr-release assays, the percentage spontaneous lysis was $24\% \pm 3\%$ for M-1, $23\% \pm 3\%$ for M-2, $24\% \pm 2\%$ for M-14 and $23\% \pm 2\%$ for LCL-14.

CD4+ T cell clones were considered cytotoxic if lysis was greater than 20% at an E:T ratio of 40:1 in the 16-h assay.

Cytokines. Human recombinant IL-2 (specific activity 3×10^6 Cetus units or 18×10^6 IU/mg) was kindly provided by the Cetus Corporation, Emeryville, Calif. Human recombinant IL-4 (specific activity 1.5×10^7 U/mg) was a gift of Sterling Drug Inc., Malvern, Pa. Human recombinant IFNγ (specific activity, 20×10^6 U/mg) was generously provided by Genentech Inc., South San Francisco, Calif.

Melanoma cells were incubated with 100 U/ml IFNγ for 24 h [2]. The IFNγ was removed from the melanoma cells by washing three times before the assay.

Flow cytometry. For detection of MHC and accessory molecules on tumor cells by indirect immunofluorescence, target cells were labeled with anti-HLA-class-I (mAb W6/32), anti-HLA-class-II (mAb L227), anti-CD54 (ICAM-1) and anti-CD58 (LFA-3) at 4°C for 30 min. The cells were washed and restained with the $F(ab')_2$ fraction of sheep anti-(mouse IgG)-FITC (Sigma, St. Louis, Mo.) for another 30 min at 4°C. Flow cytometry was then performed by FACS. Mouse mAb for human CD58 (LFA-3), HLA-A,B,C (mAb W6/32) and HLA-DR (mAb L227) were obtained from the supernatants of the hybridomas (American Type Culture Collection, Bethesda, Md.). Mouse mAb to human CD54 (ICAM-1) were purchased from Amac Inc., Westbrook, Me.

Blocking studies with mAb. Mouse mAb against HLA-A,B,C (mAb W6/32), HLA DR (mAb L227), and CD4 (mAb OKT4), were used for studies on the blocking of cytolysis by T cells. A mAb specific for human CD3 (mAb OKT3) was used as a control for the maximal inhibition of T cells achievable under these circumstances. All mAb were harvested from the supernates of their respective hybridomas (American Type Culture Collection, Bethesda, Md.). The concentrations of the mAb were measured by an enzyme immunoassay, using mouse IgG as a standard (Sigma). The concentrations of mAb used were 10 μg/ml for preincubation experiments, and 2.5 μg/ml when the mAb were present throughout the ^{51}Cr-release assay. The above concentrations of mAb were considered the optimal doses on the basis of previous experiments in our laboratory. In the experiment shown in Table 7 no mAb was added. Target cells were preincubated with either mAb W6/32 or mAb L227 for 45 min at 4°C prior to the cytotoxicity assay. T cells were preincubated with mAb to CD3 or CD4 at 4°C for 45 min.

Results

Generation of CD4+ T cell clones

A total of 25 CD4+ T cell clones were isolated from PBL or tumor tissue by limiting dilution from mixed lymphocyte/tumor cell cultures (MLTC), with either allogeneic (M-1) or autologous (M-14) melanoma cells as stimulator cells. The M-1 melanoma cell line was used to amplify melanoma-reactive T cells because it was a component of the melanoma theraccine administered to the patients under study. Most of the clones were derived from one patient (WB). Thirteen clones were isolated from the peripheral blood after stimulation with either allogeneic M-1 (6 clones: WB25, 49, 50, 53, 54, 55) or autologous M-14 melanoma cells (7 clones: WB44, 46, 68, 69, 72, 73, 75). An additional 8 were cloned from a TIL culture in the presence of autologous M-14 melanoma cells (WB80, 81, 83, 86, 87, 91, 94, 95). Finally, 4 other CD4+ T cell clones (CC6, GW5, 6, and RB1) with similar characteristics were derived from the PBL of three other patients (CC, GW, and RB) after restimulation in vitro with allogeneic M-1 melanoma.

Lysis of melanoma cells by CD4+ cell clones

Short-term (6-h) ^{51}Cr-release assays did not detect lysis (less than 5% at E:T ratios of 20:1 and 40:1) against either autologous or allogeneic melanoma cells except for 1 clone, WB44. However, cytotoxicity was detected by extending the incubation period to 16 h (Tables 2 and 3).

With a long-term incubation, cytotoxicity against the stimulating melanoma cells was detected in a significant proportion of the CD4+ T cell clones. The lytic activity of 9 of the 10 clones expanded with allogeneic M-1 melanoma cells and of 15 clones isolated after autologous stimulation is summarized in Tables 2 and 3 respectively. Of 9 clones generated after allogeneic MLTC, 5 (GW5, 6, WB25, 55, 49) killed M-1 melanoma cells efficiently, with lysis ranging between 28% and 42% at an E:T ratio of 40:1 (Table 2, columns 1–3). Patient WB had HLA alleles A2 and C3 in common with melanoma M-1. Since patient GW had no MHC class I molecules in common with M-1 target cells, it is probable that we were measuring alloreactivity (resulting from her previous immunization with that tumor cell line) with her clones rather than true melanoma reactivity.

The 10th clone isolated after stimulation in vitro by allogeneic M-1 melanoma cells, CC6, was cytotoxic to M-1 (66% lysis at E:T of 40:1) as well as the HLA-class-II-*negative* melanoma M-2 (79% lysis at E:T of 40:1). Since we did not test this clone as extensively as the others, it was not included in Table 2. Clone CC6 was not cytotoxic to the Daudi B cell lymphoma cell line, and thus does not have the properties of a lymphokine-activated-killer-type cell. The ability of the CD4+ clone to lyse target cells lacking HLA class II indicated that its recognition of the melanoma cells was independent of the presence of the HLA class II molecule. However, patient CC shared HLA class I alleles HLA-A2, HLA-B60 and HLA-C3 with melanoma M-1 cells and HLA-A28 and HLA-B60 with M-2.

Of the 15 clones isolated from the blood or TIL of patient WB after autologous MLTC, 5 (WB68, 72, 73, 80, 83) were cytotoxic to autologous M-14 melanoma cells, with lysis ranging from 21% to 37% at a ratio of 40:1 (Table 3). None of those clones lysed the HLA-identical LCL-14. By this criterion, all were considered to be melanoma-specific, as were 2 clones (WB25, 55) generated by allogeneic MLTC (Table 2). We could not determine whether the cytotoxicity detected against melanoma cells M-1 was melanoma-cell-specific because we were unable to produce the corresponding LCL from lymphocytes from the M-1 donor.

The remaining 4 clones of 9 generated in allogeneic MLTC were noncytotoxic, as were 10 of 15 clones isolated after an autologous MLTC.

Table 2. Melanoma reactivities of CD4+ T cell clones after allogeneic mixed lymphocyte/tumor cell culture MLTC against melanoma cell lines and a MHC-matched lymphoblastoid cell line: enhancement of lysis by pretreating M-1 melanoma cells with interferon γ (IFNγ)

Clones	Lysis (%)[a]									
	Target cells								Target cells after IFNγ[b]	
	M-1		M-2		M-14		LCL-14		M-1	
	20:1[c]	40:1[c]	20:1	40:1	20:1	40:1	20:1	40:1	20:1	40:1
WB25	30	28			16	31	0	0	49	48
WB55	21	28			19	27	0	0	41	36
GW5	40	28			4	10	0	0	75	65
GW6	24	38			0	1	0	0	22	59
WB49	31	42			14	19	0	0	36	87
RB1	13	18			5	9	0	19	45	50
WB50	2	7			3	3	0	0	35	42
WB53	4	4			0	0	0	0	30	39
WB54	11	9			0	1	0	0	30	43
CC6	65	66	66	79						
GW1	24	29	32	37	0	0				

[a] Percentage lysis of target cells after a 16-h incubation period. A clone was considered cytotoxic if lysis of the M-1 cell line was equal to or greater than 20% at the E:T ratio of 40:1

[b] M-1 melanoma cells were cultured with 100 U/ml IFNγ for 24 h prior to being used as targets in the cytotoxicity assay. The cells were washed extensively to remove any residual IFNγ

[c] E:T ratio

Table 3. Cytotoxicity of CD4+ T cells cloned after autologous MLTC: effect of pretreatment of melanoma cells with IFNγ

Source of lymphocytes	Clones	Lysis (%)[a]							
		Target cells		Target cells after IFNγ[b]					
		M-14		M-14		M-1		LCL-14	
		20:1[c]	40:1[c]	20:1	40:1	20:1	40:1	20:1	40:1
PBL	WB68	20	37	45	70	15	30	10	15
	WB72	24	36	66	86	19	21	12	14
	WB73	18	29	31	38	14	20	6	9
	WB44	0[d]	11[d]	20[d]	19[d]	0[d]	0[d]	3[d]	5[d]
	WB69	12	19	31	42	8	14	2	2
	WB46	7	10	16	25	0	0	0	5
	WB75	5	13	20	24	6	6	9	12
TIL	WB80	15	21	29	63	8	21	3	6
	WB83	19	26	36	44	10	12	5	5
	WB94	1	4	29	39	22	26	0	0
	WB95	0	7	27	37	9	23	6	11
	WB81	10	15	17	30	4	5	4	1
	WB86	4	9	17	31	0	1	8	11
	WB87	5	10	21	33	4	2	9	7
	WB91	2	3	13	20	2	1	10	12

[a] Percentage lysis of target cells after a 16-h incubation period. A clone was considered cytotoxic if lysis of the M-14 cell line was at least 20% at an E:T ratio of 40:1

[b] M-14, M-1 and LCL-14 cells were cultured with 100 U/ml IFNγ for 24 h prior to use as targets in the cytotoxicity assay. The cells were washed extensively to remove any residual IFNγ

[c] E:T ratio

[d] Lysis was measured after a 6-h incubation period

Sensitization of melanoma cells to lysis by pretreatment with IFNγ

Since IFNγ has been shown to up-regulate the expression of MHC antigens and melanoma-associated antigens in a variety of cell lines [2, 3, 8, 11, 25], we evaluated the ability of these CD4+ T cell clones to lyse IFNγ-pretreated melanoma and the other target cells. IFNγ increased the sensitivity of both M-1 and M-14 to lysis by CD4+ T cell clones known to be cytotoxic, as well as to those that were previously noncytotoxic to untreated target cells (Table 2, columns 8–9; Table 3, columns 5–10). In these experiments, IFNγ was removed by washing prior to the cytotoxicity assay. Pretreatment of LCL-14 cells with IFNγ did not

Table 4. The effects of IFNγ on HLA, CD54 (ICAM-1) and CD58 (LFA-3) expression on melanoma and lymphoblastoid (LCL) cells

Cell line	IFNγ	HLA class I		HLA class II		ICAM-1		LFA-3	
		MFI[a]	+ve cells (%)	MFI	+ve cells (%)	MFI	+ve cells (%)	MFI	+ve cells (%)
M-1	–[b]	859	99	567	34	675	100	582	97
	+	937	100	572	87	662	100	587	96
M-14	–	793	96	534	16	569	99	618	95
	+	840	99	611	93	720	99	619	98
LCL-14	–	678	81	709	89				
	+	669	98	663	81				

[a] MFI, mean fluorescence intensity
[b] Targets were pretreated with either medium (– or IFNγ 100 U/ml for 24 h (+)

Table 5. Analysis of HLA class I and class II restriction of 7 autologous melanoma-specific CD4+ clones

Clones	Lysis (%) of M-14 melanoma cells incubated with:							
	Control		Anti-HLA-I		Anti-HLA-II		Anti-CD3	
	20:1[a]	40:1[a]	20:1	40:1	20:1	40:1	20:1	40:1
WB46	16	25	3 (81)[b]	18 (28)	13 (19)	24 (4)	6 (63)	13 (48)
WB81	17	30	7 (59)	15 (50)	17 (0)	21 (30)	8 (53)	18 (40)
WB94	29	39	12 (59)	21 (46)	25 (14)	34 (15)	12 (59)	24 (63)
WB95	27	37	11 (59)	23 (38)	22 (19)	39 (–5)	8 (70)	25 (32)
Mean	22±3	33±3	8±2(65)	19±2(41)	19±3(13)	30±4(11)	8±1(61)	20±3(46)
WB83	36	44	23 (36)	20 (55)	22 (39)	24 (45)	26 (28)	40 (9)
WB86	17	31	11 (35)	18 (42)	11 (35)	16 (48)	0 (100)	4 (87)
WB87	21	33	16 (24)	32 (3)	14 (33)	28 (15)	3 (86)	8 (76)

[a] E:T ratio
[b] Values in parentheses show percentage inhibition relative to lysis in the absence of mAb

make them susceptible to lysis by melanoma-specific CTL clones. However, LCL-14 was not intrinsically insensitive to lysis. Lysis was achieved even in 4-h ^{51}Cr-release assays with alloreactive CD4+ cytotoxic T cell clones as well as alloreactive CD8+ T cell clones [15]. In all, 16 clones were found to be cytotoxic to autologous M-14 pretreated with IFNγ but not to similarly pretreated LCL-14, including 2 clones generated from allogeneic MLTC.

Table 4 summarizes the expression of HLA class I, HLA class II, CD54 (ICAM-1) and CD58 (LFA-3) molecules on three target cell lines. HLA class I molecules were constitutively expressed by all M-1 and M-14 melanoma cells (99% and 96%, respectively). Pretreatment with IFNγ did not significantly alter the level of expression, as indicated by the mean fluorescence intensity (M-1: from 859 to 937; M-14: from 793 to 840). In contrast, IFNγ increased the percentage of cells expressing HLA class II antigens as well as their level of expression by both melanoma cell lines. Treatment with IFNγ did not affect the level of expression of CD54 and CD58 on either cell line, where they were already maximally expressed.

HLA class I and II restriction of CD4+ T-cell-mediated cytotoxicity

Cytotoxic CD4+ T cells were reported by others to recognize and kill target cells in an HLA-class-II-restricted fashion [4, 7]. However, as we have noted, clone CC6 was cytotoxic to an HLA-class-II-negative allogeneic melanoma cell line, indicating that recognition, at least by this clone, was restricted by other molecules.

Therefore, we tested the ability of mAb to HLA class I, HLA class II and CD3 to block the lysis of autologous melanoma M-14 by 7 autologous CD4+ T cell clones. In these blocking studies, the melanoma cells were always pretreated with IFNγ.

The results of experiments with 7 individual clones are shown in Table 5. The lysis of autologous melanoma cells M-14 by 4 clones (WB46, 81, 94 and 95) was significantly inhibited by anti-HLA class I mAb, which reduced lysis from a mean of 33% (±3% SE) to 19% (±2% SE) at an E:T ratio of 40:1. However, anti-HLA-class-II mAb did not affect lysis (30%±14% SE). As expected, anti-CD3 control mAb inhibited lysis, reducing it to 20% (±3% SE). The blocking effects at the E:T ratio of 20:1 were similar to those at the E:T ratio of 40:1. The percentage of inhibition by the mAb to HLA class I mAb and CD3 was greater at an E:T ratio of 20:1 than at 40:1 (65% inhibition versus 41% inhibition).

The results of blocking studies on the remaining 3 clones (WB83, 86 and 87) are presented in the second portion of Table 5. Interestingly, lysis of 2 clones (WB83 and 86) was inhibited by both anti-HLA-class-I and -II mAb. For reasons that are still unclear, clone WB83 was

Table 6. Blocking by mAb to HLA class I of the lysis of allogeneic melanoma M-1 mediated by 4 CD4+ T cell clones

Clones	Lysis (%) of M-1 melanoma cells incubated with:									
	Control		Anti-HLA-I		Anti-HLA-II		Anti-CD3		Anti-CD4	
	20:1[a]	40:1[a]	20:1	40:1	20:1	40:1	20:1	40:1	20:1	40:1
WB50	35	42	19 (46)[b]	21 (50)	29 (17)	32 (24)	23 (34)	15 (64)	5 (86)	5 (88)
WB53	30	39	7 (77)	18 (54)	41 (–37)	44 (–13)	14 (53)	16 (59)	10 (67)	12 (69)
WB54	30	43	10 (67)	16 (63)	36 (–2)	45 (–5)	15 (50)	18 (58)	17 (43)	21 (51)
Mean	32±2	41±1	12±4(63)	18±2(56)	35±3(–7)	40±4(2)	17±3(46)	16±1(60)	11±4(65)	13±5(69)

[a] E:T ratio
[b] Values in parentheses show percentage inhibition relative to lysis in the absence of mAb

Table 7. Lysis of autologous melanoma M-14 by CD4+ T cell clone WB44: effects of preincubation of target cells with mAb in a 6-h assay

Lysis (%) of M-14 cells preincubated with:									
Control		Anti-HLA-I		Anti-HLA-II		Anti-CD3		Anti-CD4	
20:1	40:1[a]	20:1	40:1	20:1	40:1	20:1	40:1	20:1	40:1
20	19	1 (95)[b]	2 (90)	17 (15)	20 (–5)	2 (90)	2 (90)	8 (60)	12 (37)

[a] E:T ratio
[b] Values in parentheses show percentage inhibition relative to lysis in the absence of any mAb

the only clone in this series not inhibited by the mAb to CD3. We verified that it was a T cell clone since it expressed messages for both the Vα and Vβ genes of the T cell receptor (data not shown). It is possible that the T cell receptor complex was not involved in killing by this clone. The last clone, WB87, was not blocked by mAb to either HLA antigen, although anti-CD3 inhibited killing by 76%.

We also tested the effects of mAb on the lysis of allogeneic M-1 by 3 CD4+ T cell clones derived from allogeneic MLTC (Table 6). Whereas the average lysis of melanoma cells was 41% in the absence of blocking mAb, anti-HLA-class-I mAb reduced this by over one-half, to 18% (±2% SE) at an E:T ratio of 40:1. Notably, anti-HLA-class-II did not affect the degree of lysis (40% ± 12% SE). Again, anti-CD3 mAb reduced lysis to 16% (±1% SE). We also found anti-CD4 mAb to be inhibitory, reducing lysis to a mean of 13% ± 5% SE. This is probably through its direct negative effect on the T cell.

In the blocking experiments summarized above, the mAb were left in the medium for the entire incubation period, and could have acted on effector cells as well as targets under these circumstances. Therefore, we repeated the experiments, pre-incubating the melanoma cells with mAb, after which they were removed by washing, and using a more conventional 6-h ^{51}Cr-release assay. Specifically, mAb to HLA class I and II molecules were incubated for 45 min with the M-14 target cells and the mAb to CD3 was incubated for the same length of time with the CD4+ clone WB44. Antibodies were removed by washing the cells twice prior to the cytotoxicity assay. Table 7 summarizes these data. Lysis of the autologous M-14 cells by clone WB44 was 19% at an E:T ratio of 40:1. That lysis was completely blocked by anti-HLA-class-I (2%) and anti-CD3 (2%) and partially blocked by anti-CD4 (12%) mAb.

Preincubation with anti-HLA-class-II mAb had no effect on lysis (20%). Clone WB44 was highly specific for the autologous melanoma cells, since it did not lyse the autologous LCL-14 (3%).

In all, these data indicated at the clonal level that some melanoma-reactive CD4+ CTL apparently recognized their target epitopes in the context of the HLA class I molecule.

Discussion

These data indicate that human CD4+ T lymphocytes have the capacity to kill melanoma cells, and that a proportion of such CTL are HLA-class-I-restricted. The potency of CD4+ CTL appeared to be less than that of their CD8+ counterparts, as judged by the necessity for ^{51}Cr-release assays longer than 4 h, and the requirement for effector:target ratios of 40:1. In the experiments reported here, treatment of the target cells with IFNγ was essential to permit optimal detection of cytotoxicity by CD4+ CTL in vitro. While IFNγ had been shown to sensitize melanoma cells to lysis by CD8+ T cells, this observation had not been extended to cytotoxic CD4+ T cell clones to the best of our knowledge. While it is possible that we were dealing with oligoclonal populations, our methods of obtaining the putative clones by limiting dilution and the single TCR sequence in at least 4 of them support our presumption of monoclonality.

Augmentation of the expression of melanoma-associated epitopes on the cell surface together with the HLA molecules presenting those antigens may be a major mechanism of action of IFNγ. In our experiments, the expression of HLA class I antigens and accessory molecules (CD54 and CD58) on untreated melanoma cell lines M-1 and

M-14 was already maximal. IFNγ produced no significant increase. Nevertheless, after treatment with IFNγ, lysis of M-1 and M-14 was increased to 52% and 40% respectively. This suggests an increased expression of melanoma-associated epitopes associated with HLA molecules on the cell surface. A similar conclusion was reached regarding the means by which IFNγ improved cytotoxicity by CD8+ CTL among tumor-infiltrating lymphocytes [3, 32]. IFNγ increases intracellular antigen processing and presentation by up-regulating genes that may encode peptide-transport proteins and proteasomes [18, 26]. Proteasomes are structures containing proteinases thought to be involved in generating peptides from cytoplasmic proteins, for presentation on HLA molecules. These data further support our hypothesis that the higher degree of lysis after the IFNγ treatment was probably due to increased melanoma epitope expression within HLA molecules on the target cells.

CD4+ CTL recognizing tumor-associated antigens presented in an HLA class I context have also been noted by Drs. F. Vanky and E. Klein (personal communication, 1991) and D. Herlyn and colleagues [31]. In particular, CD4+ CTL that lysed melanoma cell lines lacking HLA class II antigens, as well as those that lysed HLA-class-II-positive lines but were blocked only by anti-HLA-class-I mAb, have been noted with some frequency by several investigators in our laboratory. The use of IFNγ made it easier for us to detect such cells by magnifying the degree of cytotoxicity.

A *caveat* about our conclusions concerning the HLA restriction of the CD4+ CTL arises from the incomplete blocking effects on lysis that we obtained with the mAb, which were directed against public determinants on HLA class I and HLA class II molecules. We considered blocking of 40%–50% relative to baseline to be "significant" within a given experiment, when compared with the effects of anti-HLA class II, anti-CD4 and anti-CD3 mAb. The latter failed to block completely, even though we were dealing exclusively with T cells. This indicates the necessary limitations of such blocking experiments with mAb that are not against the binding site of the peptide on the HLA molecule. In the 16-h assays we were forced to use, there could have been dissociation of the mAb from the tumor cells, which might have reduced the level of blocking. For that reason, we used a short-term assay too, where the level of blocking was 90%–95%. Our conclusions from that assay were tempered by the initial level of cytotoxicity of 20% despite pretreatment with IFNγ.

If a proportion of CD4+ CTL are HLA-class-I-restricted, the way in which their T cell receptor interacts with melanoma epitopes under these various circumstances is not readily explainable by current theories. Endogenous antigens, such as tumor-associated peptides, become associated with HLA class I molecules in the endoplasmic reticulum and are then transported with the latter to the surface of the cell where they are "presented" to CTL [6]. In contrast, exogenous (macrophage-processed) antigens become associated with HLA class II molecules in the endosomes and are, therefore, presented to CD4+ (T helper) cells in a HLA class II context. We have found CD4+ T cells that can recognize endogenous epitopes in a HLA class I context and some others that see epitopes in either a HLA class I or a HLA class II context. This implies that the T cell receptor of such cells must resemble that of the CD8+ CTL. It also implies presentation of endogenous epitopes in a HLA class II context. It does not necessarily follow that the same peptides are presented in HLA class II molecules on macrophages as in HLA class I molecules on tumors. In fact, considerable data have emerged recently that underscore their dissimilarities [5, 14].

The lower cytotoxicity elicited by CD4+ T cells than by CD8+ CTL suggests a weakness of interaction between CD4+ CTL and their targets. This is probably because CD4+ cells are preferentially helper or inducer cells rather than killers. Co-recognition of HLA class I molecules is presumably not the preferred function of CD4+ cells, which have been shown to interact with macrophages bearing HLA class II molecules. Most evidence indicates that the CD4 molecule itself does not interact with HLA class I, and therefore cannot function as a co-receptor as it does in helper functions [13]. mAb against CD4 inhibited killing, here and in other work from our laboratory (Goedegebuure P. et al., submitted for publication), even with HLA-class-I-restricted CTL, but probably because the CD4 molecule can transduce negative signals into the cell [1].

The degree of lysis caused by CD4+ CTL is not only consistently less than that of CD8+ CTL, but also requires longer assay times (16 h) and higher E:T ratios. Thus, although some CD4+ and CD8+ CTL have in common HLA class I restriction, their mechanisms of tumor cell destruction may differ qualitatively. It is possible that cytokines released by CD4+ CTL account for the lysis of melanomas, either by true cytotoxicity or from cumulative cytostatic effects.

Of 11 clones studied with anti-HLA mAb, only 1 was not inhibited by either anti-HLA class I or anti-HLA class II mAb. This is similar to the behavior of one other "MHC-unrestricted" CD4+ CTL clone we have encountered in the past [9]. Vanky et al. [33] showed that after in vitro immunization with MHC-positive tumor cells, CTL lines could lyse the same adenocarcinoma cells lacking MHC antigens. Strong avidity of the T cell receptor for the antigen after hyperimmunization in vitro or in vivo could be one factor, making the additional interaction of CD and HLA molecules unnecessary.

Our data with bulk cultures of lymphocytes suggested that human CTL react more broadly to melanomas than in a self-restricted manner. CD4+ CTL, as well as CD8+ CTL [15, 16] at the clonal level do in fact have reactivity against several different melanomas. In our study, the patients from whom melanomas M-1 and M-14 were derived, and the melanoma cell lines themselves were very similar in HLA phenotype, sharing HLA-A2 and HLA-C3 alleles and perhaps B62 (Table 1). That similarity may explain the killing of both lines of melanoma cells by CD4+ CTL from patient WB. Of 6 WB CD4+ CTL clones, elicited after stimulation with allogeneic melanoma M-1 in vitro, 2 reacted against autologous melanoma M-14, while 6 WB CTL stimulated with autologous M-14 also lysed M-1. These findings support our original hypothesis in constructing an allogeneic vaccine that there are antigens common to melanomas that can stimulate reactivity against autochthonous tumors.

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