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Lymphocytes cytotoxic to uveal and skin melanoma cells from peripheral blood of ocular melanoma patients*

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Summary. To study antitumor immunity in patients with choroidal melanoma, T cells were generated from the peripheral blood of choroidal melanoma patients by mixed lymphocyte/tumor cell culture (MLTC). Because autologous tumors are generally unavailable, an allogeneic choroidal melanoma cell line, OCM-1, was used as the specific stimulus. Lymphocyte cultures from 27 patients were characterized by cell-surface phenotypes, patterns of reactivity towards cells of the melanocytic origin and T-cell-receptor gene usage. Antimelanoma reactivity was found in cell-sorter-purified CD4+ and CD8+ T cell subsets. To analyze this reactivity, sorter-purified CD4+ and CD8+ cells from a MLTC were cloned by limiting dilution in the presence of exogenous interleukin-2 and interleukin-4 as well as irradiated OCM-1. Under these conditions, CD4+ T cells did not proliferate, perhaps because of the absence of antigen-presenting cells. However, CD8+ grew vigorously and 29 cytolytic CD8+ T cell clones were isolated. On the basis of their pattern of lysis of OCM-1, a skin melanoma cell line M-7 and its autologous lymphoblastoid cell line LCL-7, the clones were categorized into three groups. Group 1, representing 52% of the clones, lysed all three target cells, and are alloreactive. However, since OCM-1 and M-7 did not share class I antigens, these clones recognized cross-reactive epitope(s) of the histocompatibility locus antigen (HLA) molecule. Group 2, constituting 28% of the clones, lysed both the ocular and skin melanoma cell lines but not LCL-7, and were apparently melanoma-specific. Unlike classical HLA-restricted cytolytic T lymphocytes, these T cells might mediate the lysis of melanoma cells via other ligands or a more degenerate type of HLA restriction. For the latter, the HLA-A2 and -A28 alleles would have to act interchangeably as the restriction element for shared melanoma-associated antigen(s). Group 3, representing only 10% of the T cell clones, was cytotoxic only to OCM-1, but not to M-7 or LCL-7. These clones may recognize antigens unique to ocular melanoma cells. Our data suggest that choroidal melanoma patients can recognize melanoma-associated antigens common to both ocular and cutaneous melanoma cells, and presumbly their autologous tumor. Thus, choroidal melanoma, like its skin counterpart, may be responsive to immunotherapeutic regimens such as active specific or adoptive cellular immunotherapy.

Key words: Cytotoxic T lymphocytes – Melanoma – Mixed lymphocyte/tumor culture

Introduction

Melanoma of the uveal tract is the most common potentially fatal primary intraocular disease in adults [3, 16, 49]. Both clinical and laboratory data suggest that uveal melanoma is recognized as being immunogenic by the patients. Primary lesions can remain localized for long periods [37] and metastatic disease is frequently observed decades after enucleation [46]. Indeed, rare spontaneous regressions have been documented [17, 24]. In murine models of ocular melanoma, T cells and their subsets as well as other lymphoid cells can reject primary tumors [29] and metastases [20, 38]. In humans, a specific delayed-type hypersensitivity reaction (DTH) was induced in patients immunized with an antigenic extract prepared from allogeneic tumor tissues [9]. Preparations of melanoma antigens inhibited the migration [11, 42] and proliferation [50] of leukocytes from these patients.

The ability to expand human lymphocytes with recombinant interleukin-2 (IL-2) has facilitated their study. Clinical trials such as active specific immunotherapy [5, 35, 36] and adoptive transfer of tumor-infiltrating lymphocytes

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(TIL) [45, 54] further suggest that antitumor immunity can be modulated for objective clinical responses. It is now well-accepted that T cells are central to the immune response to tumors in humans.

Initially, the in vitro analysis of the host/tumor interaction, on the basis of bulk lymphocyte populations from tumors or the peripheral blood, revealed a remarkable degree of phenotypic and functional heterogeneity in the lymphocytes mediating possible anti-melanoma responses (reviews, [21, 41]). In terms of cytolytic responses, several lymphocyte populations (T, NK and LAK cells) can contribute to the lysis of tumor cells.

Specific recognition of tumor cells by T cells was demonstrated at the clonal level. When autologous tumor cells were used as the stimulus, the cytolytic activity was directed mainly to the autologous tumor cells or to allogeneic tumor cells of the same histological type [27, 41]. For cutaneous melanoma, clonal analysis of cytolytic T cells revealed heterogeneity in terms of phenotype, specificity, patterns of reactivity on allogeneic cells and mechanisms of interactions [21, 41]. However, only a small proportion of the T cell clones were specific to autologous melanoma in a *classical* self-major histocompatibility-complex (MHC)-restricted, T cell-receptor-dependent fashion. The majority of the T cell clones are not restricted in their lytic pattern to autologous melanoma and do not interact with tumor target cells in a classical MHC-restricted fashion. Recent studies on the fine specificity of T cell clones revealed that specific cytotoxic T lymphocytes can lyse allogeneic tumors sharing histological origin and at least one allefe of the class I histocompatibility locus antigen (HLA) with the autologous neoplastic cells [13, 47].

In this study, we have examined the feasibility of generating tumor-reactive T cells from the peripheral blood of choroidal melanoma patients in a mixed lymphocyte/tumor cell culture (MLTC) induced by an allogeneic choroidal melanoma cell line, OCM-1. An allogeneic melanoma cell line was used as a specific stimulus because autologous tumors from these patients are rarely available for laboratory studies. Indeed, in contrast to metastastic skin melanoma, choroidal melanoma cell lines have been particularly difficult to establish and are relatively rare [25]. Our results indicate that tumor-specific T cells thus obtained recognize melanoma-associated antigens expressed on tumors from different patients. The interaction of these T cells with their target cells may be restricted in a nonclassical MHC-restricted fashion.

Materials and methods

Tumor cell lines and melanocyte culture. The origin and characterization of the choroidal melanoma cell line, OCM-1, in our laboratory have been previously described [18]. The other melanoma cell lines were derived in our laboratory from metastatic lesions biopsied from patients with cutaneous melanoma. These were shown to be melanomas by immunochemical staining for vimentin and S100 as well as for the presence of premelanosomes, the latter was also confirmed by ultrastructural analyses. HLA typing was kindly performed by Dr. P. I. Terasaki at the University of California at Los Angeles, Calif. K562 and Daudi cell lines were obtained from the American Type Culture Collection, Md. All tumor cell lines were cultured in RPMI medium (Gibco, Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum. The human foreskin melanocyte culture was a generous gift from Dr. Ruth Halaban at Yale University, New Haven, Conn. These were grown in Ham's F-10 medium supplemented with 5% fetal calf serum, 85 nM 12-*O*-tetradecanoylphorbol-13-acetate, 0.1 mM isobutylmethylxanthine (Sigma Chemical Corp., St. Louis, Mich.), 20 μg/ml bovine pituitary extract as a source of b-fibroblast growth factor and 5 μg/ml insulin [18]. All cultures were tested negative for mycoplasma contamination every 6 weeks by Hoechst staining for cytoplasmic DNA.

Patients. A group of 27 individuals with clinically confirmed localized choroidal melanomas were entered onto this study. They were all outpatients in good health. Eleven patients were untreated for this disease. Ten were enucleated at different times prior to study. One of these individuals had measurable residual disease. Five patients received iodine-125 brachytherapy while 1 was treated with laser irradiation. Thus, these 6 individuals had residual disease (Table 1).

Mixed lymphocytes/tumor cell cultures. Mononuclear cells (MNC) were isolated from heparinized peripheral blood of choroidal melanoma patients immediatedly after venipuncture by centrifugation on Ficoll/Hypaque. Cytotoxic T cells were expanded in vitro in mixed lymphocyte/tumor cell cultures (MLTCs) containing 2×10^6 MNC and 10^5 irradiated (40 Gy) OCM-1 cells/ml in HEPES-buffered RPMI medium supplemented with 10% human AB serum and 20 Cetus IL-2 units/ml. OCM-1 was selected because it contained melanoma cells with all the major morphologies defined by the Callender classification [25]. A low concentration of IL-2 was used to support the expansion of T cells without inducing high levels of lymphokine-activated killer activity.

After 7–9 days in culture, the effector cells were harvested and assayed for cytotoxicity to OCM-1 cells. The cultures were re-stimulated with irradiated OCM-1 at weekly intervals and fed with fresh medium containing IL-2. After an additional 7–10 days, the cytolytic activities of these effector cells were analyzed against a variety of cell types.

All cultures were initiated from fresh mononuclear cells. None of the lymphocytes was cryopreserved prior to study.

Determination of cytotoxicity. Cytotoxicity assays were performed in 96-well round-bottomed microculture plates in a total volume of 200 μl. Each well contained 2×10^3 ^{51}Cr-labeled target cells. The effector cell:target cell (E/T) ratios used were 100/1, 50/1, 25/1 and 12.5/1 for assays involving a 6-h incubation. For experiments in which effector cells used had to be sorted first for CD4$^+$ or CD8$^+$ expression, a 19-h incubation was used to measure cytotoxicity at the lower E/T ratios of 10/1, 5/1, 2.5/1 and 1.25/1. To estimate the amount of released ^{51}Cr, 100 μl supernate was harvested and counted in an automated gamma counter. Percentage cytotoxicity was calculated as 100× (^{51}Cr in test well − spontaneous release) / (maximal release − spontaneous release), where ^{51}Cr release is measured as cpm.

Lymphocytes were considered cytotoxic if lysis was greater than 10% at an E/T ratio of 100/1 for the 6-h and 10/1 for the 16-h assays.

Flow cytometry and cell sorting. The surface CD phenotypes were determined by flow cytometry within 3 days of the time cytotoxicity assays were performed. The Simultest Reagent used for T and natural killer (NK) cells was a mixture of fluorescein-isothiocyanate(FITC)-conjugated anti-Leu4 (CD3) monoclonal antibody (mAb) and phycoerythrin(PE)-conjugated anti-Leu11c (CD16) and anti-Leu19 (CD56) mAb. That used for CD4 and CD8 cells was FITC-conjugated anti-Leu3a (CD4) mAb with PE-conjugated anti-Leu2a (CD8) mAb (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). All mAbs were used according to the manufacturer's specifications.

To label MNC for cell sorting, 10×10^6 MNC were incubated for 30 min on ice with saturating amounts of the appropriate Simultest Reagent. After being washed twice with medium containing 1% human AB serum, the cells were separated by simultaneous double-color cell sorting on FACStar (Becton Dickinson). Gates were set to exclude dead cells and fluorochrome-labeled cells were sorted on the basis of narrow forward-angle light scatter and fluorescence intensity into two mutually exclusive subsets of CD4$^+$/CD8$^-$ and CD4$^-$/CD8$^+$ cells. Reanalysis of

Table 1. Lymphocyte subpopulations and cytotoxic activities of the mixed lymphocyte/tumor cell cultures derived from patients with ocular melanoma. Cytotoxicity was evaluated at an E/T ratio of 100/1

Clinical status	Lysis of OCM-1 (%)	T:NK		CD4:CD8	
		T, NK (%, %)	(ratio)	CD4, CD8 (%, %)	(ratio)
Plaqued, residual disease	10	66,10	(6.6)	56,22	(2.5)
	20	71,14	(5.1)	52,22	(2.6)
	28	58.20	(2.9)	60.12	(5)
	45	55,22	(2.5)	57,15	(3.8)
	32	60.19	(3.2)	54,23	(2.3)
Laser, residual disease	20	80,16	(3.0)	59.20	(2.9)
Untreated, small to large primaries	0	81, 8	(10)	69,16	(4.4)
	6	85, 7	(12,3)	61,31	(2)
	13	78,10	(7.8)	69,48	(1.2)
	21	77,12	(6.4)	77,16	(4.8)
	22	74,14	(5.3)	67,15	(4.5)
	24	85, 6	(14)	70.25	(2.8)
	25	60, 3	(20)	53,30	(1.8)
	26	66, 7	(9.4)	59,27	(2.2)
	30	83, 9	(9.2)	55,16	(3.4)
	41	67,10	(6.7)	56,25	(2.2)
	45	65, 8	(8.1)	38,44	(0.9)
Enucleated, NED[a]	1	84, 9	(9.5)	79,12	(7.8)
	6	82, 2	(41)	69,24	(5.5)
	6	78, 4	(19.5)	77,14	(5.5)
	7	76, 3	(25.3)	84, 6	(14)
	50	66,26	(2.5)	53,25	(2.1)
	71	63,22	(2.9)	45,35	(1.3)
	20	38,33	(1.1)	35,33	(1.1)
	50	35,55	(0.6)	33,27	(1.4)
	77	29,52	(0.6)	22,15	(1.5)
Enucleation, residual disease	34	77,14	(5.5)	85, 9	(9.4)

[a] NED, no evidence of disease

sorted cell populations revealed that the purity of the respective subsets was always greater than 95%.

Prior to evaluating their cytotoxic activity, sorted CD4+ and CD8+ lymphocytes were allowed to recover by an overnight incubation in complete culture medium at 37° C.

Limiting-dilution cultures. The following protocol was used to expand purified CD4+ and CD8+ T cells. Graded numbers of CD4+ or CD8+ responder cells were cultured in 96 replicates in round-bottom microtiter plates with 1000 irradiated OCM-1 melanoma cells per well (40 Gy). The culture medium (200 µl well) was identical to that used for MLTC with the added supplement of 100 Cetus U/ml IL-2 (Cetus Corp., Emeryville, Calif.) and 10 ng/ml of IL-4 (Sterling Drug Inc., Malvern, Pa.). All microtiter wells were cultured in a humidified atmosphere of 5% CO_2 in air. The cultures were fed with 100 µl fresh medium with lymphokines and irradiated OCM-1 every week. Clones were expanded by splitting cells from one into three microwells.

T-cell-receptor(TcR) gene rearrangement analysis. To determine the extent of TcR gene usage by T cells in the MLTC, we examined the diversity of Vα and Vβ genes in transcripts of rearranged TcR α and β chain genes. We exploited gene amplification by the polymerase chain reaction. This method allows enzymatic amplification of a target DNA sequence. By selection of the appropriate oligonucleotides, the method is sensitive enough to detect a DNA sequence from one T cell in a population of 10^5 cells within a solid tumor mass [28].

To analyze the usage of Vα genes, we analyzed the cDNA reverse-transcribed from mRNA isolated from the MLTCs. Eighteen different Vα and 21 Vβ-specific oligonucleotides representing the major human TcR Vα or Vβ families were used for the 5′ primers and a Cα or Cβ sequence was used for the 3′ primer [6, 10, 39, 40, 59]. Total RNA was extracted from each MLTC and was reverse-transcribed according to established procedure [32]. The resulting cDNA was amplified using individual sets of Vα-Cα or Vβ-Cβ primers or with primers for actin for polymerase chain reaction control. The polymerase chain reaction cycle profile was 95° C denaturation for 1 min for 35 cycles, and products were separated on 2% standard agarose gels and blotted to the nitrocellulose membrane. Expression-rearranged Vα and Vβ families were considered positive when a rearranged band was hybridized with horseradish-peroxidase-labeled Cα and Cβ probe by Southern blotting [32, 56].

Results

Generation of cytotoxic lymphocytes by mixed lymphocyte/tumor cell cultures (MLTC)

Of the 27 MLTC, 21 (78%) were cytotoxic to OCM-1, lysing 10%–77% of the target cells at an E/T ratio of 100/1 (Table 1). Six cultures were noncytotoxic to OCM-1 (less than 10% lysis). Between 76% and 85% of the lymphocytes in these cultures were CD3+ T cells, the majority of which were of the CD4+ helper phenotype. Indeed, we were unable to elicit lytic activity in these cultures with additional coculture with OCM-1 for 4 weeks.

All of the cultures exhibited minimal NK and lymphokine-activated killer (LAK) activities, lysing both K562 and Daudi cells (between 20% and 35% at an E/T ratio of 100/1).

Flow cytometric analysis of the effector cells

To determine whether T or NK/LAK lymphocytes might be primarily responsible for this cytotoxicity, flow cytometry of these cultures was performed (Table 1). Although all of the MLTCs were heterogeneous, CD3+ T cells predominated in the majority of the cultures (24 of the 27). In general, 55%–85% of the lymphocytes were CD3+ T cells, with only 2%–22% NK/LAK cells. The percentage of NK/LAK cells was much higher only in 3 of 9 cultures derived from enucleated patients. In these cases, NK/LAK cells ranged from 33% to 55% of the total cell number, only 29%–38% being T cells. Most cultures contained more CD4+ T cells than CD8+ cells; the CD4/CD8 ratio ranged from 0.9 to 14.

A statistically valid association between the clinical status of the patients and the acquisition of cytotoxic activity to OCM-1, the percentage of T cells, or one of its subsets, could not be drawn from these few data. Nonetheless, cultures derived from patients with active disease appeared to have higher ratios of T cells to NK cells (CD3+ versus CD16+ CD56+). A more detailed analysis of this as well as other specific anti-tumor immunity may be useful for this purpose.

Specificity of reactivity against melanomas and melanocytes

To identify the predominant T cell immune reactivity in the MLTCs, the lytic pattern of bulk cultures against other melanomas and against normal melanocytes was examined. Ten MLTCs with more than 80% CD3+ T cells were included. Table 2 summarizes their reactivities to

Table 2. Lysis of melanoma cell lines and a melanocyte culture by mixed lymphocyte/tumor cell cultures (MLTC). Cytotoxicity was evaluated at an E/T ratio of 100/1

Pt.	clinical status[a]	Lysis (%) of				
		OCM-1	M-1	M-2	M-7	Melanocytes
TH	l	70	40	43	30	0
PH	p	24	34	31	27	0
ES	e	49	47	43	25	11
HA	e	77	67	57	87	20
RG	s	78	39	15	50	30
WP	s	19	10	–	–	0
ME	s	46	43	28	–	0
DH	m	56	21	–	–	6
ML	p	75	35	–	–	13
JW	p	40	28	–	–	59

[a] p, plaque-treated; s/ml, small/medium/large primaries; e, enucleation

Table 3. Usage of T cell receptor (TcR) Vα genes in MLTCs

Patient	TcR Vα families[a]
MW	1, 2, 3, 5, 7, 9,10,11,12, 16,17,18
NW	1, 2, 3, 5, 7, 8, 9, 11,12,13,14,15,16,17,18
MG	1, 2, 3, 4, 5, 7, 8, 9,10,11,12,13,14, 16,17,18
RB	1, 2, 3, 5, 7, 8, 9, 12,13, 16,17,18
LB	2, 5, 7, 8, 9,10,11,12,13,14,15,16,17,18

[a] Expression of Vα families was considered positive when rearranged amplified polymerase chain reaction products (300–420 bp), visualized by eithidium bromide staining, were positively hybridized with horseradish-peroxidase-labeled Cα-specific probe

Table 4. Usage of TcR Vβ genes in MLTCs

Patient	TcR Vβ families[a]
MW	1, 2, 3, 4, 5.1,5.2, 6, 7, 8, 9, 11,12,13,14,15, 17,18,19,20
NW	1, 2, 3, 4, 5.1,5.2, 6, 7, 8, 9,10,11,12,13,14,15, 17,18,19,20
MG	1, 2, 3, 4, 5.1,5.2, 6, 7, 8, 9,10, 12,13,14,15, 18,19,20
RB	1, 2, 3, 4, 5.1,5.2, 6, 7, 8, 9,10,11,12,13,14,15, 17,18,19,20
LB	1, 2, 3, 4, 5.1,5.2, 6, 7, 8 ,9,10,11,12,13,14,15, 17,18,19,20

[a] Expression of Vβ families was considered positive when rearranged amplified polymerase chain reaction products (300–420 bp) were positively hybridized with Cβ-specific probe

OCM-1, 3 allogeneic skin melanoma cell lines (M-1, M-2 and M-7) and normal melanocyte. All 10 cultures lysed between one and three other melanoma cell lines in addition to OCM-1. In contrast, 5 of the 10 cultures were not cytotoxic to normal melanocytes (TH, PH, WP, ME, and DH). Of the other 5 cultures, 4 (ES, HA, RG, and ML) lysed melanocytes but to a lesser degree than melanoma cells. This does not appear to be due to an intrinsic resistance to lysis by the melanocytes, since they were efficiently lysed by the JW culture. To show that lysis of OCM-1 was mediated via the TcR/CD3 complex, the CD3+ (T cells) and CD3− cells of 3 cultures were separated by fluorescence-activated cell sorting (FACS). Lysis of OCM-1, mediated only by CD3+ T cells, was blocked by anti-CD3 monoclonal antibody (10 μg/ml) (data not shown). Reactivity against K562 and normal melanocytes was not observed.

Table 5. Lysis of OCM-1 mediated by cell-sorter-purified CD4+ and CD8+ T cells. Cytotoxicity was evaluated at an E/T ratio of 20/1 after 16 h incubation

Patient	Clinical Status	Lysis (%) of OCM-1 by		
		Unsorted	CD4+	CD8+
ME	Untreated, s	46	36	46
WP	Untreated, s	19	24	33
RG	Untreated, s	78	12	74
DH	Untreated, m	56	31	73
TH	Untreated, l	70	51	63
PH	Plaqued	24	25	40
JW	Plaqued	40	15	72
ML	Plaqued	76	5	47
HA	Enucleated	77	6	52
ES	Enucleated	49	5	62
Average lysis (%)		54	21	56
Standard deviation		20.6	15.3	14.7

Table 6. Lysis of melanoma cell lines and a melanocyte culture by cell-sorter-purified CD8+ cells from mixed lymphocyte/tumor cell cultures. Cytotoxicity was evaluated at an E/T ratio of 20/1 after a 16 h incubation period

Pt	Clinical status	Lysis (%) of			
		OCM-1	M-1	M-2	Melanocytes
ME	s	46	47		
PH	p	40	43		
ES	e	63	61		0
HA	m	77	67		20
JW	p	72	50		39
WP	s	33	16		0
DH	m	73	20		17
TH	l	63			31
WJ	1Rx	38	37	12	43
LB	e	49	29	11	54

[a] p, plaque-treated; s/m/l, small/medium/large primaries; 1Rx, laser-treated

T-cell-receptor (TcR) gene rearrangements in MLTC

Rearrangements of the TcR genes can be used to define clonality involving T cell responses [51]. Analysis of Vα and Vβ expression in 5 MLTCs are shown in Tables 3 and 4. Almost all subfamiles were amplified from cDNA samples obtained from each culture tested. Each sample showed that one or two Vα or Vβ genes were not expressed. Interestingly, no Vβ 16 transcripts were recognized among Vβ gene transcripts in all cultures, although these primers efficiently amplified phytohemagglutin-A-activated peripheral blood lymphocytes.

Cytotoxicity of T cell subsets against melanomas and melanocytes

Most mature T cells can be subdivided on the basis of surface marker expression into two mutually exclusive subsets [43]. To determine which phenotypic subset of CD3+ T cells mediated this cytotoxicity, we isolated pure populations of CD4+ and CD8+ lymphocytes from day-7 MLTCs by cell sorting and then assayed their ability to lyse

Table 7. HLA types of patient WJ and melanoma cell lines

Cells	Class I antigens			Class II antigens		
	A	B	C	DR	DRw	DQ
Patient WJ	2,24	60,62	**3**	4,13		
Melanoma cell lines						
OCM-1	24,**28**[a]	35,62	**3**	–	–	–
M-1	**28**	60	**3**,6	4,10	53	8
M-2	**28**,31	51,60	6	–	–	–
M-7	2,11	5,52	4,5	–	–	–

[a] shared HLA A-allele

Table 8. Expansion of CD8+ lymphocytes from patient WJ for clonal analysis. Cytotoxicity and cell-surface CD phenotypes at various times after initiation of MLTC

Time in of culture (days)	Lysis of OCM-1 (%)				T,NK (%,%)	CD4,CD8 (%,%)
	100	50	25	12.5		
9	20	17	10	6		
16	67	41	26	11	81,26	59,20
35[a]	93	79	63	38	100, 0	0,99

[a] MLTC was initiated on days 0; CD8+ cells were sorted on day 17

Table 9. Lysis of melanoma and other cell lines by polyclonal CD8+ cells after culture in interleukin-2 and interleukin-4. Cytotoxicity was evaluated at 4 E/T ratios for a 16 h incubation period

E:T	OCM-1	M-1	M-2	M-7	Melano-cytes	Daudi	K562
10/1	28.2	37.0	12.1	32.0	42.9	0.0	17.0
5/1	22.9	37.1	13.2	30.4	39.0	2.4	11.5
2.5/1	15.9	25.1	4.6	25.7	25.4	0.9	7.3
1.25/1	8.8	14.6	4.0	15.5	10.0	0.0	1.3

OCM-1 in vitro. As shown in Table 5, in the majority of the cultures (8 of 10), lysis was mediated by both CD4+ and CD8+ subsets. In 3 cultures, only CD8+ cells were cytotoxic. Overall, the CD8+ populations appeared to be more cytotoxic than their CD4+ counterparts at a specific E/T ratio.

The sorted CD8+ cytotoxic T cells also lysed other melanomas and, to various degrees, melanocytes. Our data on 10 cultures are presented in Table 6. The pattern of reactivity appeared to parallel that of the original unsorted lymphocyte cultures. A similar analysis on CD4+ cells could not be performed because an insufficient number of these CD4+ cells were recovered after sorting.

Expansion and characterization of cytotoxic CD8+ T cells

The lysis by MLTCs or their sorted CD8+ T cells of more than one allogeneic melanoma cell line (Tables 2 and 6) argues against an exclusively alloreactive response. To examine this in more detail, the melanoma cell lines were HLA typed and the data are presented in Table 7. Since no B-locus alleles were common between OCM-1 and any other targets, alloreactive T cells induced by MLTC with OCM-1 would not recognize any of the B alleles. Similarly, alloreactivity specific for the C3 allele in the C locus would not lead to lysis of M-2 or M-7. As for the A locus, T cells specific for either the A-24 or the A-28 allele should not lyse the M-7 melanoma cell line. However, cytotoxic T cells can recognize an immunodominant epitope shared by multiple class 1 MHC molecules [33]. Since the A-28 and A-2 alleles are highly homologous, it is possible that T cells might be (a) restricted by the A2/28 determinant or (b) alloreactive to that determinant, accounting for the cytotoxicity towards the three melanoma cell lines. These data alone do not distinguish between these two possibilities. For this reason, we proceeded to analyze the cytotoxicity at the clonal level.

The following culture conditions were developed to expand pure populations of T cells from MLTC. Peripheral blood MNC from a patient (WJ) were co-cultured as usual with OCM-1 and IL-2. Cytotoxicity to OCM-1 was studied on days 9 and 16 (Table 8). As usual, the culture was found to be progressively more cytotoxic to OCM-1. Phenotypic analysis on day 16 showed the cells to be predominantly CD3+ T cells, 20% of which were also CD8+. On day 17, highly purified CD8+ and CD4+ lymphocytes were isolated by cell sorting. The T cell subsets were then re-cultured in 96-well round-bottomed microtiter plates at limiting dilution conditions without further addition of irradiated mononuclear cells as antigen-presenting cells. A higher level of IL-2 (100 U/ml) was added to the medium and IL-4 (10 ng/ml) was also included to promote preactivated T cell growth [48] as well as to inhibit IL-2-induced LAK activity [27]. Irradiated OCM-1 melanoma cells (500/well) was added every week as a specific stimulus. The lymphocytes were fed with fresh medium at least once a week.

Under these conditions, CD8+ T cells grew exponentially for up to 2 months, doubling every 36 h. In contrast, CD4+ T cells did not proliferate very well and were not studied. This was true for lymphocyte cultures derived from three other patients. Since CD4+ T cells have been thought to be restricted by class II molecules [51], the class-II-negative OCM-1 melanoma cells might require antigen-presenting cells to stimulate the CD4+ T cells.

The cytotoxic capacity of the expanded polyclonal CD8+ culture was not limited to OCM-1. Table 9 summarizes the results on the four allogeneic melanoma cell lines, a melanocyte culture, and the Daudi and K562 cell lines. As with CD8+ cells derived from other patients that were assayed immediately after sorting (Table 6), these polyclonal CD8+ T cells were cytotoxic to OCM-1 and the other allogeneic melanoma cell lines, although least to M-2. In this case, the melanocytes were also lysed. In contrast, the LAK target cell Daudi was not lysed at all, while the NK target cell K562 appeared to be lysed to a small degree (10%). Since K562 is particularly prone to spontaneous lysis, especially in the 16 h assay, this level of chromium release may not represent specific killing. The surface phenotype of the T cell culture was very stable and was confirmed to be CD3+ CD8+ on day 45.

Table 10. Lysis of OCM-1, M-7 and LCL-7 by CD8+ T cell clones

Group number	Clone designation	E:T	Lysis of OCM-1	Lysis (%)	
				M-7	LCL-7
1	2	9	+	5	16
	7	21	+	44	28
	8	23	+	28	26
	9	5	+	38	24
	10	12	+	21	23
	13	10	+	40	27
	20	10	+	33	22
	22	16	+	14	20
	23	13	+	22	20
	26	17	+	30	19
	27	30	+	4	14
	29	10	+	4	11
	32	9	+	3	12
	42	13	+	31	13
	44	32	+	32	27
2	1	19	+	11	0
	6	7	+	24	0
	16	20	+	18	0
	19	26	+	20	0
	11	10	+	18	2
	21	10	+	26	0
	33	7	+	18	1
	36	9	+	14	5
3	3	37	+	0	0
	14	22	+	4	0
	28	5	+	0	0
	48	13	+	4	2
	47	9	+	9	3
	49	5	+	9	4

Pattern of cytotoxicity of CD8+ T cell clones

A total of 29 CD8+ T cell clones were isolated from two microtiter plates seeded at 3 cells/well. Thus, approximately 5% of the sorter-purified CD8+ cells gave rise to clones. All of these were cytotoxic to OCM-1. Complete lysis of irradiated OCM-1 added to stimulate the growth of these clones was observed after 24–36 h under phase-contrast microscopy.

To characterize this cytotoxicity, the clones were tested against the M-7 melanoma cell line and its autologous lymphoblastoid cell line, LCL-7 (Table 10). On the basis of their pattern of lysis, we categorized the T cell clones into three groups. The first group (group 1, Table 10), representing 52% of the clones (15 of 29), appeared to be alloreactive T cells. Most were cytotoxic to all three cell lines, consistent with the recognition of MHC element(s) shared by OCM-1, M-7 and LCL-7. Surprisingly, four clones (2, 27, 36 and 39) actually lysed the LCL-7 line preferentially. The second group (group 2, Table 10), constituting 28% of the clones (8 of 29), were highly cytotoxic to M-7 but not to LCL-7. This lytic activity resembled that of melanoma-specific cytotoxic T cells induced by autologous melanoma cells that killed both autologous and allogeneic melanoma cells [1, 15, 22, 44, 55]. Thus, these results confirmed and extended our previous findings on bulk cultures that melanoma-specific CD8+ T cells were expanded in vitro by allogeneic melanoma cells [35, 36]. Lastly, the third group (group 3, Table 10), representing the smallest percentage of the clones (20%, 6 of 29), failed to lyse either M-7 or LCL-7, although they were cytotoxic to OCM-1. These T cell clones might recognize a tumor epitope or a MHC molecule expressed by the ocular melanoma OCM-1 but not the skin melanoma M-7 or the corresponding lymphoblasts. Precedence for recognition by cytotoxic T cells of heterogeneity within a broad histotype of tumor has been described [2].

Discussion

To study the cell-mediated immune responses to tumor-associated antigens in choroidal melanoma patients, an allogeneic ocular melanoma cell line was used to stimulate T cells in mixed lymphocyte/tumor cell cultures. Allogeneic tumor cells were used because the autologous tumors and/or their cell lines were not available with this type of tumor. In fact, cytotoxic T cells specific for both autologous and allogeneic melanomas have been described [21, 41].

Cytolytic T cells (CTL) were identified in the majority of the allogeneic MLTCs. Lysis was mediated by both CD4+ and CD8+ lymphocyte subsets. CD4+ lymphocytes cytotoxic to human tumor cells have been reported in lymphocyte cultures derived from patients with a variety of malignant diseases, including melanoma of the skin [19, 23, 34]. In general, the CD4 antigen is thought to be involved in the process of antigen recognition and probably serves as an associative recognition structure of the MHC class II antigen [23]. Since OCM-1 does not express class II antigens, the CD4+ T cells described here may mediate lysis of OCM-1 via another ligand or recognition structure [19, 21, 34, 53]. However, we were unable to induce the proliferation of these CD4+ T cells without adding irradiated autologous mononuclear cells as a source of antigen-presenting cells.

Since the specificity of T cells arises from rearrangement of the TcR Vα and Jα genes, an Vβ-Dβ and Jβ genes, analysis of TcR gene usage was performed in 5 early MLTCs. We found T cells in the early MLTCs to be highly polyclonal, although Vβ-16 transcripts were not identified. We have previously shown a predominance of Vα-7 transcripts in TILs from ocular melanoma tissues [39]. It is interesting that this transcript was also detected in all MLTCs.

Proof for the existence of melanoma-specific CTL was provided at the clonal level for the CD8+ lymphocyte subset. Because analysis of the TcRs revealed polyclonal T cells in MLTCs between days 18 and 20, we studied the lytic patterns of CD8+ clones after a brief period of expansion, in an attempt to capture all possible specificities. None of the CD8+ T cell clones exhibited LAK activity, although this type of nonspecificity has been described for CD3+ CTLs after long-term culture in exogenous lymphokines [7].

The T cell clones in group 1 were alloreactive, lysing all three target cell lines (Table 10). They appear to recognize an epitope common to both HLA-A2 and A28 loci. Cross-reactivity to class I antigens has also been demonstrated in

mixed lymphocyte cultures [52]. However, this type of reactivity is rare [30]. In contrast, the majority of the group 1 CTL clones were cross-reactive. The presence of the melanoma peptide(s) in association with the MHC molecules of OCM-1 may have favored their expansion. Alternatively, it is possible that these T-cell clones may have "dual specificity", recognizing MHC–melanoma-antigen complexes that coincidentally resemble allogeneic molecule(s). Dual-specific, class-I-restricted, influenza-specific cytotoxic T cell clones that recognize one or more uninfected allogeneic target cells have already been described [58].

The T cell clones in group 2 exhibited an interesting melanoma-specific reactivity. They lysed OCM-1 as well as M-7, while sparing LCL-7. Since the two melanoma cell lines do not share any class I antigen (A24,28 vs A2,11), they differ from classical MHC-restricted CTLs. Recognition might be mediated via another structure or ligand [53], or a tumor-associated antigen, such as the large glycosylated mucin molecule expressed by pancreatic and breast tumors [4].

Alternatively, lysis of melanoma cells by group 2 CTLs could still be MHC-restricted, although it would be a more degenerate version of recognition. An example of this would be T-cells that lysed autologous as well as HLA-A2 or -A10 matched allogeneic melanomas [12, 14, 47, 57]. Furthermore, these T cells could be generated by a "simulated autologous" MLTC with HLA-A2-matched allogeneic melanoma cells [12]. The HLA-A antigens of group 2 CTLs were A2,24 while that for OCM-1 were A24,28. Since the HLA-A2 and -A28 antigens were 96% homologous in their protein sequences [31], they might be used interchangeably as the restriction element by these melanoma-specific CTLs. An A2/A28-based CTL system has not been observed before [12], and we hope to study this phenomenon by establishing long-term T cell clones with this specificity.

The T cell clones in group 3 failed to lyse the M-7 or LCL-7 besides OCM-1. It is possible that some may be alloreactive and highly specific for the HLA phenotype of OCM-1. However, others may be tumor-reactive, or even specific for ocular melanoma with or without HLA restriction. Indeed, while uveal and cutaneous melanomas express many of the same differentiation antigens, serological studies revealed the overall surface phenotype of uveal melanoma to be different from skin melanoma [8]. In fact, there is suggestive evidence that for one tumor-associated antigen, the S100 protein, the ocular melanoma polypeptide may be a variant from that expressed by skin melanoma and other neural crest tissues [26].

From a clinical perspective, these results are encouraging to those who would use immunological treatments against ocular melanoma. A proportion of choroidal melanoma patients are clearly able to recognize melanoma-associated antigens, on both ocular and cutaneous melanoma target cells, and presumably their autologous tumor. Active specific immunotherapy with either ocular melanoma antigens, or more likely with more abundant cutaneous melanoma cells, thus seems possible. Furthermore, the ability to expand in vitro both $CD4^+$ and $CD8^+$ CTLs from the peripheral blood, a proportion of which were reactive against melanoma-associated antigens, encourages the additional possibility of adoptive immunotherapy. The growth of cloned T cells, which we have shown here was feasible, may obviate the need for harvesting and expansion of tumor-infiltrating lymphocytes, even though the latter begin with an intrinsic restriction to tumor-associated epitopes [45, 54].

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