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Research Article

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Generation and Characterization of Two Human α/β T cell Clones

Recognizing Autologous Breast Tumor Cells through an HLA- and TCR/CD3-Independent Pathway

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Abstract

Cell-mediated immune response to breast tumor has only been marginally investigated. To gain insight into this issue we have developed two clones of distinct phenotype, $CD3^+$ α/β, $CD4^+$, $CD8^-$, $CD16^-$, and $CD3^+$ α/β, $CD4^-$, $CD8^+$, $CD16^-$, respectively, from peripheral blood lymphocytes (PBL) of a breast cancer patient. These effectors, selected on the basis of their cytolytic activity against autologous tumor cells and lack of lysis on NK-sensitive cell lines, preferentially recognize autologous tumor cells. The two clones' cytotoxic activity, while inhibited by anti-LFA-1 mAb, could not be abolished by mAbs to CD3, to class I and class II MHC molecules, and by mAbs to molecules involved in T cell function (i.e., CD4, CD8, CD2).

The molecular structure of the α and β T cell receptor chains of the two effector cells, confirmed their clonality and showed that, despite an overlapping killing pattern, they possess distinct TCR α and β chains. These findings demonstrate that breast tumor-specific CTL clones can be generated through current technology and that a α/β effector cell population operating through a HLA-unrestricted and TCR/CD3-independent pathway may be involved in the identification and killing of this tumor. (*J. Clin. Invest.* 1994. 94:1426–1431.) Key words: breast cancer • autologous tumor • cytotoxic activity • MHC unrestricted • TCR/CD3 independent

Introduction

Isolation and characterization of immune cells with autologous tumor reactivity have been described in a variety of solid tumors (1–6). It has been demonstrated that at least some human malignancies elicit a cell-mediated immune response and that cytotoxic T lymphocytes (CTL)[1] with autologous tumor reactivity may offer the possibility of adoptive immunotherapy as an alternative to IL-2–activated effectors. Indeed experimental evidence has been provided, showing that tumor specific CTL either derived from peripheral blood lymphocytes (PBL) or tumor-infiltrating T lymphocytes (TIL) are more efficient than lymphokine-activated killer (LAK) cells in mediating the regression of established neoplasia (7, 8). Furthermore, the functional characterization of these CTL, represents an ideal model for gaining further insight into the molecular mechanisms involved in tumor cell identification and killing.

To explore breast cancer, which in this context has been poorly investigated (9, 10), we have isolated a number of CTL clones, by coculture of PBL with autologous tumor cells. While the majority of these effectors lacked autologous tumor specificity or displayed NK-like activity, two clones of distinct phenotype preferentially lysed the autologous target. Serological evidence and sequencing of the α/β TCR chains have demonstrated that the recognition pathway of the two clones is HLA unrestricted and TCR/CD3 independent.

Methods

Cell and cell lines. Peripheral blood lymphocytes (HLA-A1,29; -B7,12; DR1,7) were obtained from heparinized blood of a 58-yr-old female patient (CCM) affected by metastatic breast cancer and free of chemotherapy for 6 mo. The patient's tumor cells were harvested from heparinized metastatic ascitic effusion. Briefly, after centrifugation at 160 *g* for 10 min, the cell pellet consisting of approximately 90% viable tumor cells (11) was resuspended in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated serum (FBS; Gibco) and 2 mM L-glutamine (Gibco). Tumor cells were propagated in culture in the same medium and the adherent population assayed immunocytochemically with a panel of mAbs to tumor-associated antigens (TAA), as previously described (11). Aliquots of cells were cryopreserved in RPMI 1640 (40%), FBS (50%), and DMSO (10%).

Autologous EBV-transformed B lymphocytes were obtained using the supernatant of the EBV-producing marmoset cell line B95-8 [American Type Culture Collection (ATCC); Rockville, MD].

The cell lines listed in Table I were employed as targets in ^{51}Cr assays. The melanoma cell lines 1402/R, 665/1, 4405 were kindly provided by Dr. G. Parmiani (Istituto Tumori, Milano, Italy) (12–13). The breast adenocarcinoma cell lines DAL, PLC, and DSN, the ovarian carcinoma MRC and the melanoma BRN were established in our laboratory as previously described (11, 14). Cell lines KJ29 (15) and Colo 38 (14) were a gift of Dr. R. Gambari (University of Ferrara, Italy) and Dr. S. Ferrone (New York Medical College, New York). The additional cell lines were obtained from ATCC, all of which were maintained in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. Mycoplasma contamination was monitored by an enzyme immunoassay kit (Boehringer, Mannheim, Germany).

Generation and isolation of CTL clones. Mixed lymphocytes tumor cells culture (MLTC) was carried out in RPMI 1640 (25 mM Hepes

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; D, diversity; J, joining; MLTC, mixed lymphocytes tumor cell culture; PBL, peripheral blood lymphocytes; TCR, T cell receptor; V, variable.

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buffer) (GIBCO), supplemented with 10% FBS and 2 mM L-glutamine, in 24-well plates (Falcon Labware, Becton Dickinson, Plymouth, England). Briefly 10^6 PBL of patient CCM were cocultured with irradiated (10,000 rad) autologous tumor cells at 5×10^5 cells in 2 ml of medium in the presence of 10 U/ml of rIL-2 (Boehringer). After 1 wk, responder cells were cloned by limiting dilution, as reported (16). Briefly, the cells (0.3/well) were plated in Terasaki plates (Falcon Labware) in the presence of irradiated autologous tumor cells (10^5/ml), allogeneic PBL (4×10^5/ml), 0.1 μg/ml PHA (Murex Diagnostics, Dartford, England) and 10 U/ml rIL-2 in a final volume of 20 μl. After 10 d of culture in a 5% CO_2 humidified atmosphere at 37°C, the growing clones were transferred to round bottom 96-well plates (Costar, Cambridge, MA) and were finally expanded in 24-well plates (Costar) employing allogeneic PBL as feeders, rIL-2 (10 U/ml) and PHA (0.1 μg/ml).

The clones were cultured under these conditions and periodically restimulated using the same protocol. Clones were employed as effectors in cytotoxicity assays 2 wk after restimulation.

Only those effector cells displaying cytolytic activity against autologous tumor cells (i.e., cytotoxicity > 20%) and no lytic activity against K562 cell line (cytotoxicity < 10%) were selected and maintained in culture.

Monoclonal antibodies. mAbs OKT3, OKT4, OKT8, and OKT11 recognizing the CD3, CD4, CD8 and CD2 molecules, respectively, were from ATCC; anti-CD3 mAb (IgM) was a generous gift of Dr. O. Acuto (Pasteur Institute, Paris) (17).

mAb Leu-11b (to CD16), mAb Leu-19 (to CD56), and mAb WT31 (to TCR α/β) were obtained from Becton-Dickinson (Mountain View, CA). mAb TCRδ1 was purchased from T Cell Sciences (Cambridge, MA). Anti-CD1a L404, anti-CD1b L0249 mAbs were described in Oxford (1987) leukocyte typing workshop and have been kindly provided by Dr. T. Hercend (IGR, Villejuif, France). Anti-LFA-1 (CD11a) mAb was a generous gift of Dr. W. Knapp (Institut für Immunologie Universitat, Wien, Austria). W6/32 mAb is directed to a monomorphic epitope of HLA-class I molecule (ATCC) (18), mAb Q5/13 directed towards class II DR and to a lesser degree class II DP molecule (19); mAb BT3/4 (20) recognizes class II DQ molecules and mAb B7/21 directed towards class II HLA-DP molecules (21), were generous gifts from, respectively, Dr. S. Ferrone (New York Medical College, New York), Dr. G. Corte (Universita di Genova, Italy), and Dr. I. S. Trowbridge (Salk Institute, La Jolla, CA). Anti TCR Vβ21 family mAb was kindly donated by Dr. F. Romagne (Immunotech, Marseille, France). The $F(ab')_2$ fragment of affinity purified OKT3 mAb was obtained by digestion with pepsin (22).

Indirect immunofluorescence (IIF) and cytofluorography analysis. IIF was performed according to standard procedures incubating 5×10^5 cells with the optimal dilution of each purified mAb, staining with affinity purified FITC-labeled $F(ab')_2$ of goat anti-mouse Ig serum (Cappel; Organon Teknika Corp., Turnhout, Belgium) and the percentage of fluorescence-positive cells was evaluated by FACS® analysis (FACScan; Becton Dickinson) (23).

Cytotoxicity assay. Cytotoxicity activity of CTL was determined using a conventional ^{51}Cr-release assay as previously described (24). Maximum release was measured by incubating labeled ^{51}Cr target cells in 2% Nonidet P-40, whereas spontaneous release was determined by incubation in culture medium. Percent lysis was calculated as follows: [(Experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Only tests in which the spontaneous release was lower than 25% were evaluated. All determinations were made in triplicate and results were reported as mean±SE (which never exceeded 10%).

Cold-target competition assay. The assay was carried out in round bottom plates adding two different numbers of unlabeled (cold) competitors to a constant number of ^{51}Cr-labeled target cells, in a volume of 150 μl. The cell mixture was then added to effector cells (50 μl) and a standard 4-h cytotoxicity assay was performed as described above.

Blocking assays of CTL-mediated lysis by mAbs. Blocking activity of mAbs was performed as follows: effector cells were incubated 1 h at 37°C with mAbs specific to CD3 and other T cell surface antigens, at the appropriate concentration ranging from 1 to 100 μg/ml. Labeled target cells were incubated with anti-MHC class I, anti-MHC class II, or anti-CD1 mAbs (10 μg/ml). Afterward effector and target cells were appropriately diluted and employed in a standard 4-h ^{51}Cr-release assay. Alternatively, effector and target cells were washed three times before plating, to remove unbound antibody, or the mAbs were directly added to the E/T cells mixture in a 4-h ^{51}Cr-release assay. Controls included incubation of antibodies with targets on their own to exclude antibody-dependent mechanisms of cell lysis in the absence of CTL. Cytotoxicity and results were evaluated as described above.

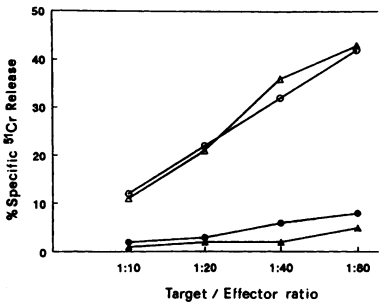

Figure 1. Autologous breast tumor-specificity of CTL clones CCM-2 ($CD4^+$) and CCM-15 ($CD8^+$). Lytic activity of CCM-2 (△, ▲) and CCM-15 (○, ●) clones were tested in a 4-h ^{51}Cr release assay against (△), (○) autologous cryopreserved breast tumor cells; (▲), (●) NK-sensitive K562 cell line, at four different E/T ratio. Data represent the mean of three experiments, performed in triplicate. SE never exceeded 10%. While the two clones induce autologous tumor cell dose-dependent lysis, no cytolytic activity is demonstrable on the NK-sensitive target.

PCR amplification and sequence analysis of TCR α and β cDNA. Isolation of total RNA, cDNA synthesis (from 5 μg of total RNA) and anchored PCR technique (A-PCR) were performed as described (25). The primers contained, respectively, a sequence (underlined) complementary to the poly (G) (5′-CACTCGAGCGGCCGCGTCGACCCC-CCCCCC-3′); and a sequence complementary to either the 5′ end of α constant region (5′-GCGAATTCAGATCTTAGGCAGACAGAC-TTGTCACTGG-3′), or the 5′ end of β-constant region (5′-GCTCTA-GAGTCGACGGCTGCTCAGGCAGTATCTGGAGT-3′). For each of the T cell clones (CCM-15 and CCM-2) two independent PCR were performed. The amplified products were directionally cloned into the sequencing vectors M13mp19, which were subsequently used to transform TG1 *Escherichia coli.* Individual plaques representing TCR α and β chains were picked. A total of 16 isolates from each PCR product were sequenced. Sequencing was performed using the dideoxy chain termination method with the Sequenase DNA sequencing kit (US Biochemical, Cleveland, OH).

Results

Phenotypic characterization of CTL clones to autologous breast tumor. From MLTCs established with peripheral blood mononuclear leukocytes and irradiated breast tumor cells (HLA-class I and II positive) of patient CCM in the presence of IL-2, 11 CTL clones were obtained by limiting dilutions. Phenotypic analysis revealed that all of them were $CD3^+$ α/β $CD4^-$, $CD8^+$, $CD16^-$, except for CCM-2 clone that showed a $CD3^+$ α/β, $CD4^+$, $CD8^-$, $CD16^-$ phenotype. On the basis of the lysis of the autologous tumor cells and absence of lysis on NK target K562 at an E/T ratio as high as 80:1 (Fig. 1), we selected for further study only CTL clones CCM-2 and CCM-15, which after further phenotypic analysis, did not display detectable CD56 molecules.

Specificity of the CTL clones CCM-2 and CCM-15. The specificity of the two CTL clones was analyzed by cytotoxicity assays on a representative panel of cell targets (Table I) which included autologous EBV-transformed B lymphocytes, alloge-

Table I. Cytotoxic Activity of CCM-2 ($CD4^+$, α/β^+) and CCM-15 ($CD8^+$, α/β^+) Clones on Cultured Tumor Cells of Various Histotype

Target Cells		CCM-2		CCM-15		HLA Phenotype
		1:20*	1:40	1:20	1:40	
Autologous						
Breast Tu	CCM	21‡	36	22	32	A1, 29, B7, 12, DR1, 7
EBV-B cells	CCM EBV	2	2	1	1	
Allogeneic tumors						
Breast	SK-BR-3	2	2	2	1	A11, BW22+/−, $B40^+$, $B18^+$
Breast	DAL	3	4	2	1	
Breast	PLC	1	2	1	3	
Breast	DSN	11	10	ND	ND	
Breast	BT20	3	3	2	1	A1, BW16+/−
Lung	A549	ND	ND	2	2	
Lung	A2182	2	3	1	2	
Lung	CALU	1	4	3	5	A10, 11, B15, W35
Renal	KJ29	36	40	26	30	
Bladder	RT4	1	1	2	2	
Ovarian	MRC	2	5	7	9	
Colon	HT-29	2	2	ND	ND	A1, 3, B12, 17, CW5
Colon	Lo Vo	3	5	3	3	A11, B15, B17, CW1, CW3
Melanoma	Colo 38	40	49	18	22	A3, 24, B35, 62, DR4
Melanoma	BRN	6	6	10	12	
Melanoma	1402/R	2	1	3	4	A3, A24, B12, B55
Melanoma	4405	3	2	5	5	A11, A24
Melanoma	665/1	2	3	5	4	A1, A24, B15, B55
Leukemia	K562	2	2	3	6	
Leukemia	KG 1	1	4	2	1	A30, 31, B35, CW4
Lymphoma	MOLT-4	10	12	10	10	

* Target/Effector ratio. ‡ Percent lysis in a 4-h ^{51}Cr-release assay. Data represent the mean of three experiments, performed in triplicate as described in Methods. SE never exceeded 10%. ND, not done.

neic breast cancer cells, and a variety of tumor cell lines of different histotype. As shown in Table I CCM-2 ($CD4^+$) and CCM-15 ($CD8^+$) clones were not cytolytic for autologous B cells and a variety of tumor cells, with the exception of the renal carcinoma KJ29 and the melanoma Colo 38 cell lines which were significantly and specifically lysed as indicated by the lack of cell killing when an unrelated $CD8^+$ CTL clone was used as effector (data not shown).

The finding that CCM-2 and CCM-15 clones also lysed Colo 38 and KJ29 cells was further confirmed by cold target competition experiments. As shown in Fig. 2, the addition of cold autologous tumor cells to the assay employing CCM-15 clone, inhibited the lysis of the hot CCM line, whereas the competitive activity of cold Colo 38 and KJ29 cells towards hot autologous cells was significantly less evident. Cold autologous cells, on the other hand, completely abolished the recognition of the other two hot targets even at a hot/cold cell ratio of 1:50 thus indicating an optimal epitope recognition of the CCM-15 CTL on the autologous target, unlikely to occur because of cell crowding. Furthermore, the inability of the K562 cell line to function as cold competitor again argues against an "NK-like" cytotoxic activity of the CTL clones. The lytic pattern of the two clones described above has remained unchanged over a period of a year.

MHC antigens and T cell surface markers are not involved in the cytotoxic activity of CCM-2 and CCM-15 CTL clones against CCM tumor cells. In order to identify the nature of the molecules involved in the cytotoxic activity of CCM-2 and CCM-15 CTL clones, we evaluated the blocking activity of a large panel of mAbs specific to functional molecules expressed on target or effector cells. Independently from the experimental protocol used (see Methods) the following results (Table II) were obtained. Anti-class I and anti-class II (DR, DP, DQ) mAbs incubated on target cells, did not block the lytic activity of CCM-2 and CCM-15 clones, indicating that the two CTL clones are likely to recognize autologous tumor in a HLA-unrestricted fashion.

The mAbs used were capable of inhibiting the lytic activity of the melanoma specific A81 CTL clone (12) on the autologous melanoma (Table II).

Knowing that CD1 molecules may be involved in HLA unrestricted cytotoxicity, we assayed two anti-CD1 antibodies (anti-CD1a and b) in blocking experiments. As shown in Table II neither reagent interfered with the CTL lytic activity. Moreover CCM-2 and CCM-15 clones were unable to kill the MOLT-4 cell line (Table I) which expresses CD1c molecules and is the target of CD1 directed, HLA-unrestricted, cytotoxicity (26). Furthermore, neither anti-TCR α/β and anti-CD3, nor anti-CD4 or anti-CD8 mAbs could block the lytic function of CCM-2 ($CD4^+$) and CCM-15 ($CD8^+$) clones, respectively. Similar results were obtained using anti-CD3 antibodies of different isotypes (IgG2a, IgM) known for their ability to inhibit CD3-

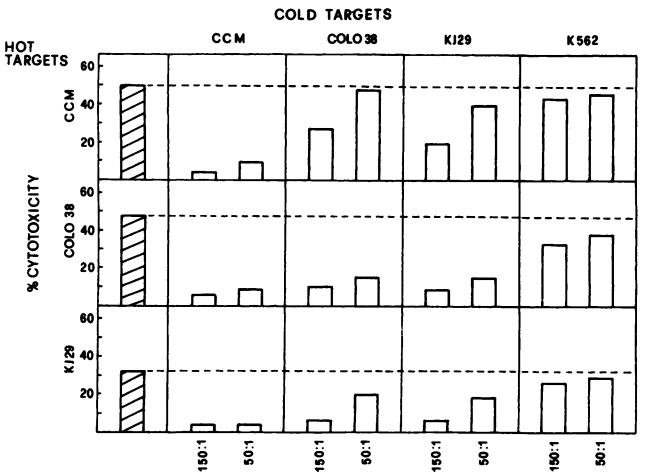

Figure 2. Cold target competition assay. The CCM-15 clone was tested at an E/T of 80:1 in a 4-h ^{51}Cr-release assay against the autologous breast tumor CCM, the melanoma cell line Colo 38, the renal carcinoma KJ29, and the K562 leukemia in the absence (■) or in presence (□) of two different concentrations (150:1, 50:1) of unlabeled (cold) competitors cell lines. While unlabeled autologous CCM cells completely block the cytotoxic activity of CTL CCM-15 on the labeled CCM, Colo 38 and KJ29 cells are only capable of a partial inhibition of the autologous tumor killing by CCM-15.

Table II. Activity of A Panel of mAbs on The Specific Lysis of The Autologous Tumor (CCM) by CCM-2 ($CD4^+$) and CCM-15 ($CD8^+$) Clones

	CCM-2	CCM-15	A81 CTL clone	
Antibody	% lysis	% lysis	% lysis −Ab	% lysis +mAb
WT31	40	38	61*	22
OKT3	38	35	"	25
Anti-CD3 (IgM)	34	30	"	23
OKT3 [F(ab')$_2$]	35	31	"	28
W6/32	40	33	61	7
Q5/13	33	30		
B7/21	36	32		
BT3/4	37	31		
OKT4	35	34		
OKT8	39	30	61	16
OKT11	33	31		
L404	32	29		
L0249	36	30		
Anti-CD11a	7	8		

CCM-2 and CCM-15 CTL clones were tested against autologous breast tumor cells in a 4-h ^{51}Cr-release assay at an E/T ratio of 40:1 in the presence of mAbs (10 μg/ml) listed above. * A81 CTL clone ($CD8^+$) was tested as control against autologous melanoma in the absence (−mAb) or in the presence (+mAb) of mAbs. Data represent the mean of three experiments, performed in triplicate. SE never exceeded 10%.

dependent lysis of tumor target (27). In order to exclude that the Fc receptor lytic effect of the whole antibody could mask the apparent absence of specific inhibition, we employed a F(ab')$_2$ fragment of anti CD3 antibody. Again we did not obtain cytotoxicity inhibition. The lytic function of CCM-2 and CCM-15 cells was significantly reduced only in presence of mAb to LFA-1. Together these data suggested that CCM-2 and CCM-15 clones are likely to recognize the tumor targets in a CD3-independent fashion.

TCR sequences of CCM-2 and CCM-15 clones. Sequencing the α and β mRNA transcripts of the CCM-2 and CCM-15 CTL clones by PCR indicate (Table III) that the CCM-2 clone expresses Vα 19.1-Jα 9.3 and Vβ 21.4-Jβ 1.1, while the CCM-15 clone expresses Vα 18.1-Jα17.10 and Vβ22-Jβ2.7. The nucleotide and deduced amino acid sequences of CDR3 regions corresponding to the 3′ end of each variable region and to the joining regions demonstrate that CCM-2 and CCM-15 are clonal populations and possess two distinct pairs of Vα/Jα and Vβ/Jβ regions and distinct CDR3 regions. Sequencing of the CCM-2 CTL clone showed that it expresses the Vβ21 family of genes as well as their product as confirmed by the finding that 98% of CCM-2 cells were positively stained (data not shown) by anti-Vβ21 mAb. On the basis of the identification of Vβ regions inhibition experiments with anti Vβ21 mAb were performed.

As reported in Fig. 3, the antibody did not block the lytic activity of CCM-2 on the three susceptible tumor targets, demonstrating the lack of functional involvement of TCR in tumor target lysis. In contrast anti-Vβ21 mAb was capable of enhancing the cytotoxicity of the CCM-2 CTL clone indicating that the clone expresses a functional efficient TCR.

Discussion

The generation of cytotoxic effector cells to breast carcinoma has rarely been reported. To the best of our knowledge a $CD8^+$ clone capable of killing autologous tumor in a HLA-restricted fashion which could be inhibited by anti-CD3 mAb has been isolated by Sato et al. (9). Noncloned CTL lines which could lyse tumor cells by a TCR-dependent but HLA-unrestricted mechanism have been reported by Jerome et al. (10).

In this study we have described the generation, from PBL of a breast cancer bearing patient, of two stable CTL clones endowed with a so far undisclosed lytic activity for the autologous tumor. Tumor target recognition, in fact, is HLA-unrestricted and lysis is CD3 independent. The identification of this population of effector cells is not unprecedented since it has already been reported in B cell lymphomas by Yssel et al. (33), but their induction by epithelial tumors has never been reported. The two clones which have been selected on the basis of their lack of NK-like cytotoxicity, express, respectively, a CD4 α/β and a CD8 α/β phenotype, but the analysis of their tumor specificity demonstrated that they possess an overlapping lytic pattern being cytotoxic for autologous tumor cells, but not for autologous EBV-transformed B lymphocytes. Furthermore, among a large panel of tumor cells of different histotype tested, they exert cytotoxic activity only on the human melanoma Colo 38 and the renal carcinoma KJ29.

On the other hand, hot/cold competition assays have confirmed that an optimal epitope recognition by the two CTL clones takes place on the autologous tumor cells. Although the antigenic specificity of the two clones is presently unknown, they do not appear to recognize mucin determinants which have been described as a stimulator and target of human breast tumor-specific CTLs (10). The two clones, in fact, while unable to lyse the mucin expressing SKBR-3 breast cancer cell line, are

Table III. Amino Acid Sequence Alignment of α and β V(D)J Regions of CCM-2 and CCM-15 CTL Clones

	Vα	CDR3	Jα	Cα
CCM-2 Vα19.1-Jα9.3	CAV	AKNSGGSNYKL	TFGKGTLLTVNP	NLQ
CCM-15 Vα18.1-Jα17.10	CAF	TTTSGTYKY	IFGTGTRLKVLA	NIQ
	Vβ	**CDR3**	**Jβ**	**Cβ**
CCM-2 Vβ21.4-Jβ1.1	CAS	SLEWSSGTEA	FFGQGTRLTVY	EDL
CCM-15 Vβ22-Jβ2.7	CAS	RTGPPYEQ	YFGPGTRLTVT	EDL

The amino acid sequences of the last three amino acid residues of each variable region, of the complete joining regions and the first three residues of the constant regions are shown in alignment. Original sequence designation of V regions is indicated in parentheses: Vα19.1 (AB21); Vα18.1 (AB24); Vβ21.4 (IGRb01); Vβ22 (IGRb03). AB21 and AB24 were described by Klein et al. (28); IGRb01 and IGRb03 were described by Ferradini et al. (29). The Jα segments were described by Moss et al. (30); Jβ segments by Toyonaga et al. (31). CDR3 regions are assigned according to Chothia et al. (32). J residues contributing to CDR3 are underlined.

cytotoxic for the mucin negative melanoma Colo 38 and renal carcinoma KJ29.

This property of the HLA-unrestricted and TCR-independent lytic mechanism of the two clones has been established on the basis of the evidence from several experiments. The lack of inhibition of cytotoxic activity by anti-class I and class II HLA mAbs and the absence of correlation of the lytic activity on a large panel of tumor targets of various HLA haplotype strongly indicate in contrast to the CD8 clone isolated by Sato et al. (9), our effector cells recognize the autologous breast tumor by an MHC-independent recognition mechanism.

Evidence that the two CTL clones are lytic through a TCR-independent mechanism has been gathered by inhibition experiments with anti-CD3 mAb and sequencing of TCR α and β chains. The latter study has disclosed that, although the two clones share an identical lytic pattern, they express different TCR α and β chains. Furthermore mAb to Vβ 21 expressed by the CCM-2 clone is unable to block its lytic activity.

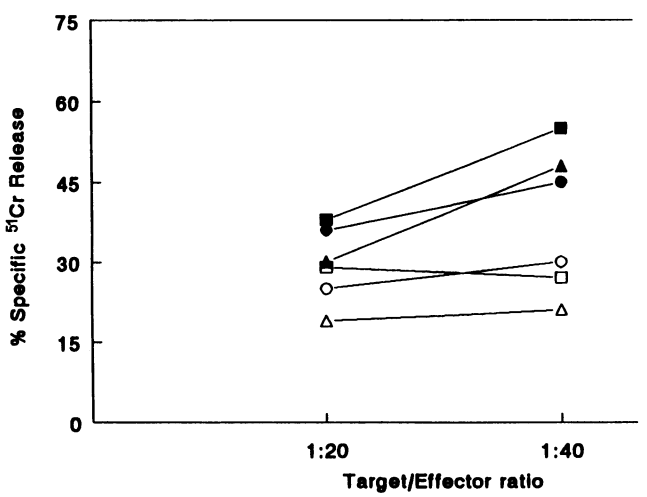

Figure 3. Cytotoxic activity of CCM-2 clone on tumor cell lines in the presence of anti-Vβ21 mAb. Lytic activity of CCM-2 clone was tested in a 4-h ^{51}Cr release assay against (□; ■) autologous tumor cells CCM, (△; ▲) melanoma cell line Colo 38, (○; ●) renal carcinoma KJ29, in the absence (□; △; ○) or presence (■; ▲; ●) of the mAb, at two different E/T ratio. Anti-Vβ21 mAb enhanced lytic activity of CCM-2 clone on the tumor targets.

These features clearly distinguish these effector cells from the noncloned CTL lines described by Jerome et al. (10), which recognize and lyse tumor cells in a TCR-dependent HLA-unrestricted fashion. This type of recognition mechanism already reported by Siliciano et al. (34) for α/β^+ lymphocytes, has been interpreted as dependent on the presence of a "nominal" antigen in a highly multivalent form able to directly bind and activate this αβ TCR, in the absence of self MHC molecules.

Further attempts to evaluate whether our clones utilize alternative recognition molecules and/or lytic pathways has suggested that CD1 and CD2 molecules may not participate. The pivotal role of the LFA-1 adhesion molecules in T cell functions (35) was confirmed by the inhibition obtained with anti-LFA-1 mAb.

In conclusion, the results of this study demonstrate that stable breast tumor-specific CTL can be generated using currently available technology. Using a screening protocol that selects tumor-specific cytotoxic T cells, we have in fact isolated and sequenced, for the first time in a breast cancer patient, the TCR α and β chains of two CTL clones with different phenotypes but the same pattern of cytotoxicity. This pattern includes a high degree of autologous tumor-specific killing which takes place through an MHC-unrestricted, CD3-independent mechanism. Although suboptimal, the ability of the two CTL clones to kill unrelated tumor cells, i.e., a melanoma and a renal carcinoma suggests that the induction of these effector cells may not be unique to breast cancer.

The identification of these clones raises the question as to whether they derive from a specific cell population or represent effector cells that employ a combination of interacting structures capable of activating the cytolytic machinery in a MHC-unrestricted fashion. Available experimental evidence indicates, in fact, that the CD3-TCR complex is unlikely to play a functional role in this type of effector-target recognition (27). Although the ability of our clones to retain their tumor specificity over a long period of time strongly suggests that they may belong to a specific effector lineage, additional experimental work is needed to confirm this hypothesis.

In view of the well known heterogeneity in the expression of MHC products and tumor-associated antigens acting as restriction elements (36), these CTL clones could represent alternative effector cells for immunotherapeutic protocols. This may be facilitated by the possibility of overcoming intratumor susceptibility to CTL lysis, as already demonstrated in a melanoma model employing CD3-independent CTL clones and anti-CD3/anti-TAA bispecific antibodies (24).

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