Identification of an Immunodominant Peptide of HER-2/neu Protooncogene Recognized by Ovarian Tumor-specific Cytotoxic T Lymphocyte Lines

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Summary

Synthetic peptide analogues of sequences in the HER-2 protooncogene (HER-2) were selected based on the presence of HLA-A2.1 anchor motifs to identify the epitopes on HER-2 recognized by ovarian tumor-reactive CTL. 19 synthetic peptides were evaluated for recognition by four HLA-A2^{+} ovarian-specific cytotoxic T lymphocyte (CTL) lines obtained from leukocytes associated with ovarian tumors. The nonapeptide E75 (HER-2, 369-377:KIFGSLAFL) was efficient in sensitizing T2 cells for lysis by all four CTL lines. This peptide was specifically recognized by cloned CD8^{+} CTL isolated from one of the ovarian-specific CTL lines. E75-pulsed T2 cells inhibited lysis by the same CTL clone of both an HLA-A2^{+} HER-2^{high} ovarian tumor and a HER-2^{high} cloned ovarian tumor line transfected with HLA-A2, suggesting that this or a structurally similar epitope may be specifically recognized by these CTL on ovarian tumors. Several other HER-2 peptides were recognized preferentially by one or two CTL lines, suggesting that both common and private HER-2 epitopes may be immunogenic in patients with ovarian tumors. Since HER-2 is a self-antigen, these peptides may be useful for understanding mechanisms of tumor recognition by T cells, immunological tolerance to tumor, and structural characterization of tumor antigens.

The existence of CTLs in the leukocyte infiltrations of ovarian tumors, that when expanded in culture in the presence of IL-2 are capable of recognizing autologous, and HLA-matched allogeneic tumors provides strong support to the hypotheses that these CTL recognize multiple private and/or common Ag on tumor and that these Ag can induce T cell responses (1, 2). A critical step towards testing this model is the identification of tumor-specific T cell epitopes. This goal is highly significant because it may lead to an understanding of the immune responses to tumors, the reasons for failure of such responses to control tumor growth in vivo, and development of novel strategies for cancer therapy. Processing of tumor cellular proteins may result in CTL epitopes. In general, the ability of peptide ligand to compete for receptor binding improves as its concentration increases, and the distinction between tumor and normal tissue reactivity may be predicated on the ability of peptides from an overexpressed protein to occupy a significant number of MHC molecules in competition with other peptides according to the laws of mass action (3). Based on these considerations, we proposed the HER-2/neu protooncogene (HER-2)[1] as a potential target for a T cell response against epithelial tumors such as those in breast and ovary, because in a number of tumors the concentration of this protein is increased by up to 100–200-fold over normal tissues. Processing of this overexpressed protein may result in increased peptide supply, which may activate/reactivate an immune response against tumor (3). In support of this hypothesis, evidence from a large case analysis in breast cancer indicates that HER-2 overexpression correlates with a favorable prognosis in patients with breast cancer having a high density of local lymphocyte infiltration (4).

The importance of HER-2 in the recognition of ovarian and breast tumors by CTL in vitro and in vivo has not yet been elucidated, nor have the common epitopes of HER-2 recognized by CD8^{+} CTL lines from different donors and cloned CD3^{+}CD8^{+}CD4^{-} CTL been identified. In this study, we have identified common immunogenic epitopes of HER-2 recognized by four out of four and two out of four CD3^{+}CD4^{-}CD8^{+} ovarian-specific CTL lines that were isolated from tumor-associated lymphocytes (TAL) from HLA-A2^{+} ovarian cancer patients. CTL clones isolated from one of these lines confirmed recognition of one common HER-2 epitope, and they suggest that a peptide with an identical or cross-reactive sequence is recognized by tumor-reactive CTL on ovarian tumors. Identification of common antigenic CTL epitopes of HER-2 may help to develop targeted immunother-

[1] *Abbreviations used in this paper:* FBP, folate-binding protein; HER-2, HER-2/neu protooncogene; MCF, mean channel fluorescence number; TAL, tumor-associated lymphocytes; TAP, peptide transporter-associated proteins; (TAP1 and TAP2); TIL, tumor-infiltrating lymphocytes.

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apeutic strategies for breast and ovarian cancer and to elucidate the mechanisms of tolerance towards these epitopes.

Materials and Methods

Synthetic Peptides and Monoclonal Antibodies. HER-2 and control peptides were synthesized by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid-phase method and purified by HPLC. Identity of the final peptides was established by amino acid analysis. The purity of the peptides used in these experiments was ≥97%. mAb to CD3 (OKT3-FITC), CD4 (OKT4-FITC), and CD8 (OKT8-FITC) were obtained from Ortho Diagnostic (Raritan, NJ), mAb W6/32(anti–HLA,-A,-B, -C) from Dako (Dakopatts, Denmark), and anti–HLA-A2 mAb BB7.2 (anti-α-2 domain) and MA2.1 (anti-α-1 domain) from American Tissue Culture Collection (ATCC, Rockville, MD). mAb *Ab2* against HER-2 was obtained from Oncogene Science (Manhasset, NY).

Target Cells and Cell Lines. The human cell line 174CEM.T2 (T2) was a kind gift from Dr. Peter Creswell (Yale University, New Haven, CT). These cells are defective in the normal antigen processing pathway and they express HLA-A2.1 occupied only by signal peptides (5). C1R transfectants C1R:A2 and C1R:A1 cells express HLA-A2.1 and HLA-A1, respectively. Parental Hmy2.C1R (class I reduced) cell line does not express any HLA-A, but expresses low HLA-B35. These cells were a generous gift from Dr. William E. Biddison (National Institute of Neurological Disorders, Bethesda, MD). Tumor lines and leukocytes of the donors of ovarian malignant ascites were phenotyped for HLA-A, -B, and -C antigens by the Blood Bank at the M.D. Anderson Cancer Center. Expression of HLA-A2 on freshly isolated ovarian tumors and transfectants was confirmed by immunofluorescence using MA2.1 mAb. Ovarian tumor lines of known HLA phenotypes, MDAH 2774 (HLA-A3, 24,B45,w57) and SKOV3(HLA-A3,28,B18(w6),35(w6), Cw5), were also used as targets in these experiments.

SKOV3 cells were transfected with the HLA-A2 expression vector RSV.5-neo containing the same full-length HLA-A2.1 cDNA expressed in C1R:A2 cells (6) (kindly provided by Dr. W. Biddison), using the Lipofectin reagent and procedure as described by the manufacturer (Gibco Life Technologies, Grand Island, NY). After selection with G418, clones that expressed high levels of HLA-A2 and HER-2 (as determined by immunofluorescence with MA2.1 and *Ab2* mAbsA) were selected for further experiments.

HER-2 Peptide Binding to HLA-A2.1 To establish the ability of HER-2 peptides to stabilize HLA-A2 expression, the T2 MHC class I peptide stabilization assay was performed as described (7). T2 cells were incubated overnight with saturating amounts of all of the selected HER-2 peptides, as well as with positive and negative control peptides at the same concentration (50 μg/ml). Cells were then washed, stained with BB7.2 and W6/32 mAbs, and analyzed by flow cytometry as described (2, 7, 8). Fluorescence intensity and positions of the peaks were determined using an Epics® V profile analyzer with a log amplifier (Coulter Electronics, Hialeah, FL). Results are expressed as the mean channel fluorescence number (MCF) on a logarithmic scale corresponding to the peak of fluorescence for HLA-A2 (8).

Generation of Ovarian-specific CTL Lines and Clones. CTL were generated by culturing freshly isolated tumor-associated lymphocytes (TAL) from ovarian malignant ascites in complete RPMI medium in the initial presence of autologous ovarian tumor, 25–50 U/ml of IL-2 (Cetus Corp., Emeryville, CA), and 250 U/ml of TNF-α (Genentech, South San Francisco, CA), for 2 wk, followed by selection of $CD8^+$ cells on anti-CD8 mAb–coated culture flasks (AIS Micro CELLector™; Applied Immune Sciences, Menlo Park, CA) and negative selection on anti-CD4 mAb coated flasks as described (9). Isolated $CD8^+CD4^-$ cell lines designated CTLs 1–4 were propagated in culture in complete RPMI medium supplemented with IL-2. $CD3^+CD8^+CD4^-$ clones were established by limiting dilution from CTL-3 line as we described (1, 2).

Identification of Antigenic Peptides. To identify the antigenic HER-2 peptides, CTL lysis of T2 cells preincubated for 60 min with each peptide was measured in a ^{51}Cr release cytotoxicity assay (1, 7). For titration of HER-2 peptides for recognition by $CD8^+$ CTL, T2 cells were incubated with varied concentrations of purified HER-2 peptides. For antibody inhibition experiments, targets were preincubated with the appropriate antibody as described (2), then washed and incubated with effectors. Percentage of specific lysis was determined from the equation $(A - B)/(C - B) \times 100$, where A is lysis of T2 cells by effectors in the presence of a peptide, B is spontaneous release from T2 cells in the presence of the same peptide but in the absence of effectors, and C is the maximum ^{51}Cr release. The experiments were performed in triplicate, and the mean ± SD values were calculated from at least two separate experiments. Since even one amino acid change in peptide length at the COOH-terminal end can have dramatic effects on peptide recognition (10), and identification of CTL epitopes is performed with synthetic peptides, cytotoxicity values were considered to indicate significant recognition of a peptide when the differences between mean ± SD values for percent of specific lysis of T2 cells preincubated with a peptide or medium were ≥10%, at an E/T ratio of 20:1 (10) and statistically significant ($p < 0.05$). Cold target inhibition of cytolysis was performed using ^{51}Cr-labeled ovarian tumors, OVA-1 (autologous with CTL-1), as well as SKOV3.A2.1E4 transfectants as "hot" targets and T2 cells pulsed with peptides as "cold" targets. T2 cells were preincubated with HER-2 or control peptides (50 μg/ml) overnight, then washed and admixed with ^{51}Cr-labeled targets at 5:1 and 10:1 (cold/hot) target ratios.

Statistical Analysis. Values obtained for percent of specific lysis by the same effectors of T2 cells preincubated with HER-2 peptides and percentage of specific lysis of T2 cells in the absence of exogenous peptides were examined by the Student's t test. Differences were considered significant when the $p < 0.05$.

Results

Identification of HER-2 Peptides That Stabilize HLa-A2.1 Expression. To identify the HER-2 epitopes recognized by these CTL, 19 peptides were selected from the HER-2 sequence based on the HLA-A2 anchor motifs based on the presence of Leu/Ile at position 2 (P2) and Val/Leu/Met at P9 (11, 12). The majority (16 out of 19) peptides selected for this study were nonamers. Two octamers were included because they were part of overlapping epitopes. 15 out of 19 peptides contained Leu (P2), while two peptides contained Ile (P2) and two peptides contained Val (P2). Only octa and decamers were found in the HER-2 sequence to contain Met at P2, and consequently these peptides were not included in the present study. Peptides were selected from signal, extracellular, transmembrane, and cytoplasmic domains of HER-2 (13). Priority was given to peptides from the regions 364-474 and 781-859 because they contain the highest density of continuous and overlapping epitopes with HLA-A2.1

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binding motifs. Peptides were also selected from the signal and transmembrane domains of HER-2 because hydrophobic, Leu-, Ile-, and Val-rich peptides were found bound on HLA-A2 of both T2 and C1R:A2 cells (5, 14), were described as CTL epitopes (10), and may be bound to HLA-A2 in transporter-associated proteins (TAP)-deficient targets (5). The peptides selected for this study represent more than 50% of all nonamers that are potential HER-2 epitopes with HLA-A2 anchor motifs (11, 12).

Since both P2 and P9 anchors and residues from the central area of peptide contact HLA-A2 and define the affinity of a peptide to the presenting molecule (12), to determine the binding ability of HER-2 peptide analogues, expression of HLA-A2 on T2 cells was determined in the presence of each HER-2 peptide and the corresponding control peptides. All peptides were tested for binding to the MHC class I in the HLA-A2 stabilization assay using T2 as indicator cells (7, 8). To establish that the results reflect MHC class I heavy chain expression indicative of absolute binding and not only the effects of peptide-induced conformational changes (8) that may affect the reactivity of mAb with the peptide-binding pocket, T2 cells were stained with both W6/32 (anti–class I monomorphic) and BB7.2 (anti–HLA-A2 α-2 domain) mAb. As shown in Table 1, 9 out of 19 HER-2 peptides (ranked 1–9 based on their HLA-A2– stabilizing ability) induced a greater than twofold increase in MCF for HLA-A2 expression, compared to negative control peptide C61, as determined with BB7.2 mAb. Similar results were obtained after staining with MA2.1 mAb (data not shown). HLA-A2 stabilization for E75, E90, and E89 was peptide concentration dependent in the range 1–50 μg/ml (not shown). This suggests that these peptides have higher stabilizing ability of both conformational epitope BB7.2, as well as HLA-A2 molecule expression than the other 10 peptides (ranked 10–19), which were designated as peptides with low stabilizing ability for HLA-A2.1. Five other peptides, E70, E71, E72, D97, and D99, did not affect serological epitope W6/32 expression, suggesting that they bound poorly to HLA-A2. Of these peptides, the octamer D97 induced a significant increase in BB.7.2 epitope expression, suggesting induction of a conformational epitope rather than stabilization of HLA-A2 expression. In contrast, BB.7.2 bound poorly to T2 cells pulsed with peptide E74 compared with W6/32. The implication of this serological analysis is that HER-2 peptides, in addition to having binding and stabilizing effects on HLA-A2 expression, may lead to conformational changes in the Ag-binding pocket.

Recognition of HER-2 Peptides by Ovarian Tumor-reactive CTL. To identify HER-2 peptides recognized by ovarian tumor–reactive CTL lines, four $CD8^+$ CTL lines designated CTLs 1–4 were generated from cultured TAL from four different HLA-A2$^+$ donors after $CD8^+$ cell selection on anti-CD8 antibody–coated plates. These CTL lines were 100% $CD3^+$, 100% $CD8^+$, and 0–2% $CD4^+$. This approach was considered necessary because the Ag specificity of $CD3^+$ $CD8^+CD4^-$ CTL isolated from tumor-infiltrating lymphocytes (TIL)/TAL, which have been in culture for 2 wk, will not be diluted or masked by the overgrowth of $CD4^+$ cells, which is encountered in long-term TIL/TAL cultures. To avoid changes in the Ag specificity, the isolated $CD3^+CD8^+$ $CD4^-$ lines were not restimulated with autologous or allogeneic HLA-A2$^+$ tumors. These CTL lines recognized autologous and allogeneic HLA-A2$^+$ ovarian tumors, but not HLA-A2$^-$ ovarian tumors or lines, as illustrated in Table 2. Since freshly isolated ovarian tumors from different donors may be antigenically heterogeneous or may express variable levels of HER-2, we needed as target a cloned ovarian tumor of high and stable HER-2 protein expression and known HLA-phenotype having HLA-A2 in common with the effectors. Tumor cells of the SKOV3 line, which overexpresses HER-2 protein (15), were transfected with the HLA-A2 gene. TAP1 and TAP2 message expression in SKOV3 is increased in parallel with HLA class I by IFN-γ treatment (16), suggesting unimpaired Ag-presenting ability. A clone SKOV3.A2.1E4 expressing high and stable levels of HER-2 protein and HLA-A2 was used as a target in these experiments. Four HLA-A2$^+$ ovarian $CD8^+CD4^-$ CTL lines lysed SKOV3.A2.1E4 clone in addition to autologous and allogeneic HER-2^{high} HLA-A2$^+$ tumors. They did not recognize HLA-A2$^+$, HER-2^{low} ovarian cell lines. Furthermore, they did not lyse K562 cells, indicating that they did not express NK or LAK activity (Table 2). Autologous tumor lysis was inhibited by mAb to CD3 TCR (OKT3) and HLA-A2 (MA.2.1), but not by anti–HLA-DR mAb, suggesting that they can recognize Ag presented by HLA-A2 (data not shown).

To evaluate whether these $CD8^+$ CTL recognized the same or different HER-2 peptides, lysis of T2 cells preincubated with each peptide was tested with all CTL lines. Both high and low affinity peptides were tested in the same experiment since it has been reported that a melanoma CTL epitope is derived from low affinity HLA-A2–binding peptides (17). For increased stringency in epitope identification, recognition of an HER-2 peptide was considered significant based on convergence of results of statistical analysis of differences in cytotoxicity data (18, 19) and assigning a cut-off value of at least 10% for the differences between recognition of T2 cells exogenously loaded with HER-2 peptides and T2 cells presenting only endogenous peptides. This approach was necessary because we wanted to identify peptides that, based on the levels of observed lysis, are either recognized with higher affinity than others or their recognition reflects the presence of a higher percentage of specific reactive clones.

Based on comparison of cytotoxicity values for T2 cells, lysis by $CD8^+$ CTL1-4 in the presence and absence of HER-2 peptides, CTL-1 and CTL-2 recognized mainly peptide E75 (Fig. 1). CTL-3 recognized, in addition to E75, three other peptides (E90, E89, and C85), but it did not recognize the remaining 15 peptides. CTL-4 recognized four of the peptides tested, including E75 and C85. CTL-4 recognized, at a lesser extent than E75 and C85, two other HER-2 peptides residues, 799-807 (E71) and 835-842 (E73). Peptides E71 and E73 were not recognized by the other three CTL lines, even when reconstitution of the epitopes was attempted at either higher peptide concentrations or higher E/T ratios, and may represent private epitopes for CTL-4 (Fig. 1). Since CTL-4

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Table 1. *Cell Surface Expression of BB7.2 Epitope on HLA-A2.1 of T2 Cells by HER-2 Peptides*

												BB7.2		W6/32	
Code	Position	1	2	3	4	5	6	7	8	9	10	MCF*	Rank‡	MCF	Rank
HER-2 peptides															
E91	5-13	A	L	C	R	W	G	L	L	L		82	9	306	8
D97	42-49	H	L	D	M	L	R	H	L			52	12	167	18
D113	48-56	H	L	Y	Q	G	C	Q	V	V		155	2	496	2
E75	369-377	K	I	F	G	S	L	A	F	L		131	3	474	4
E77	391-399	P	L	Q	P	E	Q	L	Q	V		61	10	216	11
E76	402-410	T	L	E	E	I	T	G	Y	L		109	6	358	5
E78	457-465	S	L	R	E	L	G	S	G	L		60	11	208	12
E93	466-474	A	L	I	H	H	N	T	H	L		113	5	293	9
E92	650-658	P	L	T	S	I	I	S	A	V		128	4	324	6
E88	689-697	R	L	L	Q	E	T	E	L	V		109	7	481	3
E70	793-801	T	V	Q	L	V	T	Q	L	M		35	18	172	17
E90	789-797	C	L	T	S	T	V	Q	L	V		164	1	515	1
E71	799-807	Q	L	M	P	Y	G	C	L	L		42	16	173	15
E72	828-836	Q	I	A	K	G	M	S	Y	L		32	19	166	19
E73	835-842	Y	L	E	D	V	R	L	V			51	13	234	10
E74	838-846	D	V	R	L	V	H	R	D	L		36	17	203	13
E89	851-859	V	L	V	K	S	P	N	H	V		82	8	310	7
C85	971-979	E	L	V	S	E	F	S	R	M		47	14	194	14
D99	1089-1098	D	L	G	M	G	A	A	K	G	L	44	15	172	16
Control peptides§															
HER-2															
C81	971-979	E	L	V	S	E	V	S	K	V		76		261	
C61	968-977	R	F	R	E	L	V	S	E	F	S	37		182	
Folate-binding protein‖															
E38	112-120	N	L	G	P	W	I	Q	Q	V		77		N.D.	
E37	25-33	R	I	A	W	A	R	T	E	L		34		N.D.	
E41	245-253	L	L	S	L	A	L	M	L	L		38		N.D.	
No peptide												34		172	

* Mean channel fluorescence (MCF) corresponding to the peak of fluorescence for T2 cells preincubated with 50 μg/ml of each peptide was determined for all peptides in the same experiment as described in the Materials and Methods. MCF for both W6/32 and BB7.2 are presented and compared to confirm the increase in MHC class I heavy chain expression.

‡ Peptides are ranked in decreasing order of their ability to increase HLA-A2.1 expression.

§ The variant peptide of C85 containing three substituted residues F → V(P6), R → K(P8), and M → V(P9) was used as positive control because the resulting variant (C81) contains four dominant and strong anchor residues (11, 12) reported to favorize binding to HLA-A2. The peptide C61 (HER-2: 968-977) contains HLA-B8 anchors and was used as negative control.

‖ Folate-binding protein (FBP) peptides were selected from the FBP sequence based on the concordance of T cell epitopes predicted by the computer program, ANT.Find.M (3), and the presence of HLA-A2–specific anchor motifs (11).

was isolated from the ascites corresponding to previously reported TAL-24, these results confirm the recognition of a peptide from the area 968-984 (C85) as a potential T cell epitope derived from HER-2 or a structurally similar peptide HLA-A2 complex (20). Peptide E75 was recognized by all four CTL lines, C85 by two out of four CTL lines, while peptides E89 and E90 were recognized by CTL-3, and E71 and E73 by CTL-4. All four CTL lines failed to specifically recognize a number of HER-2 peptides presented by T2 cells with canonical HLA-A2 anchors at P2 and P9 and different central sequences, including D113, which was reported to bind HLA-A2 with high affinity (12). All CTL lines showed low levels of lysis of T2 cells without exogenous peptides (T2 cells present a number of signal peptides) (5), comparable

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Table 2. *Recognition of HLA-A2⁺ Tumors by Ovarian-specific CTL Lines*

Targets‡	Percent of specific lysis*			
	CTL-1	CTL-2	CTL-3	CTL-4
Auto-T (HER-2⁺,A2⁺)	47	65	28	41
Allo-T (HER-2⁺,A2⁺)	40	41	14	38
SKOV3.A2.1E4 (HER-2⁺,A2⁺)	45	39	42	84
Allo-T (HER-2⁻,A2⁻)	5	15	NT	3
2774 (HER-2⁻,A2⁻)	0	0	0	4
K562 (HER-2⁻,A2⁻)	1	3	3	4

* Percent specific lysis is shown for an effector to target ratio of 20:1. Target lysis was determined in a 5-h 51Cr release assay. NT, not tested.
‡ Auto-T and Allo-T represent autologous and allogeneic freshly isolated ovarian tumors. SKOV3.A2.1E4 is an ovarian tumor clone expressing HLA-A2. 2774 is a human ovarian tumor line.

to lysis of the NK targets, K562 cells. These results show that ovarian tumor–reactive CTL can recognize common HER-2 epitopes, although the pattern of peptide recognition is different for each line.

Recognition of E75 by CTL-3 Clones. Results presented above are suggestive of common HER-2 peptide recognition by four distinct CTL lines. We wanted to establish whether E75 is specifically recognized by cloned CTL, and whether it may correspond to an epitope recognized by the same CTL clone on ovarian tumors that overexpress HER-2. CTL-1 and, to a lesser extent, CTL-2, appear to be highly restricted in their recognition of E75. This can cause the results of peptide specificity experiments with cloned CTL-1 and -2 to appear biased in the favor of E75. To address the question of whether reactivity to E75 was a property of distinct non–cross-reactive clones from a line of multiple HER-2 specificities, and to establish whether these clones recognize E75 in a peptide concentration–depependent and –specific manner, clones were developed from CTL-3 by stringent limiting dilution and further expanded in culture.

CTL-3 line did not lyse the C1R or C1R:A2 cells (Fig. 2 *A*). Since C1R:A2 and SKOV3.A2.1E4 were transfected with the same plasmid carrying the same HLA-A2 gene, this suggests that the endogenous peptides recognized on SKOV-3.A2.1E4 by CTL-3 may be different from the ones presented by C1R:A2 cells. Both E75 and C85 were recognized by CTL-3 and CTL-4 when presented by C1R:A2 but not by C1R:A1 transfectants (data not shown). Concentration-dependent recognition of E75, C85, and E90 was observed with CTL-3 (Fig. 2 *B*). This recognition was confirmed in independently performed experiments. Documentation of restricted expression of FBP on ovarian tumors suggests the possibility of cellular immune recognition of FBP peptides (21). CTL-3 did not recognize two peptide analogues of FBP, residues 112-120 (E38) and residues 245-253 (E41) (Fig. 2 *A*). FBP peptides were selected to include HLA-A2–binding anchors and to exhibit high (E38) and low (E41) HLA-A2– binding affinity, respectively (Table 1). Similarly, CTL-3 did not recognize two peptide analogues of the Muc-1 core peptide (22), D125 and D132. Recognition by cultured ovarian TAL of Muc-1 core exposed on ovarian tumors and of Muc-1 gene transfected and expressed by EBV-B cell lines has been suggested (23). Muc-1 core sequence lacks canonical HLA-A2 anchors at correct distances to allow binding to the main HLA-A2 pockets (21), but its recognition has been described mainly as non-MHC restricted (24). Therefore, in both Muc-1 peptides, GLTSAPDTRV (D125) and SLADPAHGV (D132), HLA-A2 anchors were introduced (underlined) to engage binding and to present the intervening sequence to TCRs. These results, together with the results presented in Fig. 1, suggest that

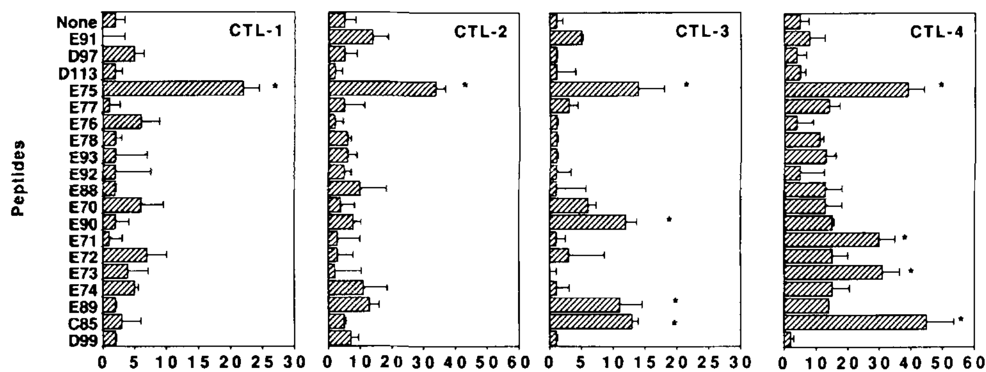

Figure 1. Recognition of HER-2 peptides by CD3⁺CD8⁺CD4⁻ CTL isolated from four different ovarian cancer patients. Cytotoxicity was determined using T2 cells preincubated for 60 min with each peptide at 25 μg/ml in a 5-h ^{51}Cr release assay. Percentage of specific lysis is shown for all CTL lines for an E/T ratio of 20:1. Percentage of specific lysis was calculated as described in Materials and Methods. Asterisk indicates mean cytotoxicity values that are at least 10 percentage points greater than mean values for the lysis of T2 in the absence of peptide and are also significantly different by Student's *t* test (p <0.05).

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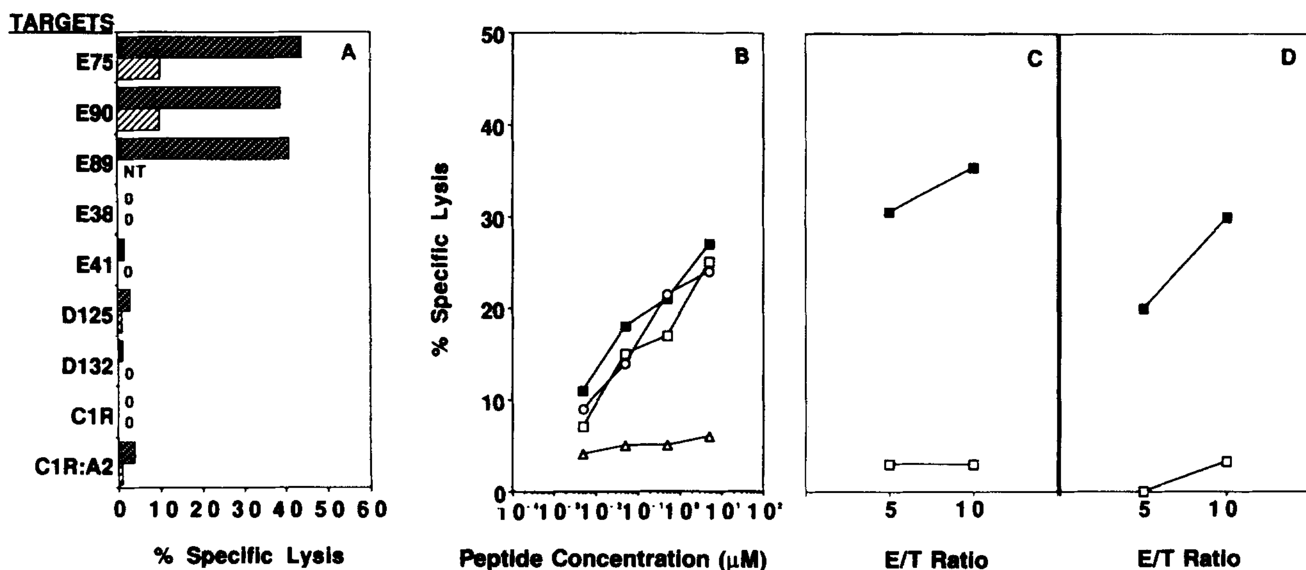

Figure 2. Recognition of E75 by CTL-3 clones. (*A*) Lysis by the CTL-3 line. 3000 ^{51}Cr-labeled T2 cells were incubated with HER-2 peptides E75, E89, and E90, FBP peptides E38 and E41, and variant Muc-1 peptides D125 and D132 at a final concentration of 25 μM for 60 min before effectors were added. Supernatant was collected and counted after 5 h. E/T ratios were 10:1 (▨) and 5:1 (■). Results are presented as the percentage of specific lysis by effectors of T2 cells pulsed with peptides. The same numbers (3,000) of C1R and C1R:A2 cells were used as targets. (*B*) Concentration-dependent recognition of E75 (■), E90 (□), C85 (○), and E92 (△) by CTL-3 line at an E/T ratio of 20:1. (*C* and *D*) Lysis by clones 3C4F (*C*) and 3B4E (*D*) of E75- (■) and E90- (□) pulsed T2 cells. Lysis of T2 cells incubated with E89 at 25 μM was 7% by clone 3C4F and 5% by clone 3B4E at 10:1 E/T ratios.

CTL-3 line contains clones that are specific for particular peptide epitopes.

Two clones, 3C4F and 3B4E, isolated from CTL-3 line that recognized E75 but not E90 or E89 peptides presented by T2 cells, are shown in Fig. 2 (*C* and *D*). Recognition of T2 cells incubated with the same concentration of E75 suggest that clones 3C4F and 3B4E are specific for peptide E75 (Fig. 2, *C* and *D*). Recognition of E75 by these two clones was compared over a range of concentrations (10 nM–10 μM) (Fig. 3 *A*). At an E/T ratio as low as 4:1, peptide E75 reconstituted T cell recognition by clone 3C4F at a concentration (100 nM) similar to that reported for an HLA-A2–restricted epitope gp100 and an HLA-A1–restricted epitope from MAGE-3 recognized by melanoma-specific CTL (25, 26), but at higher

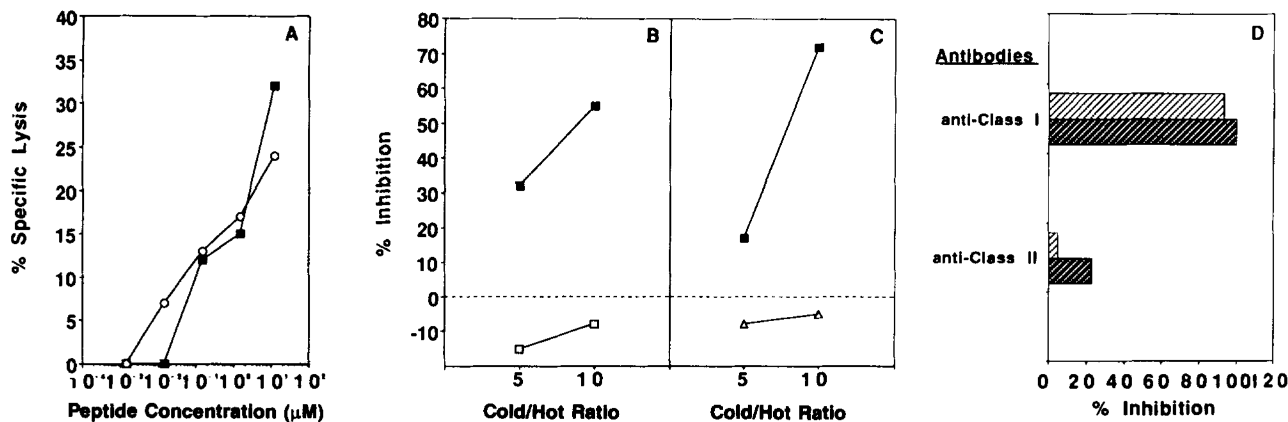

Figure 3. Inhibition of ovarian tumor recognition by clone CTL-3C4F by T cell epitope E75. (*A*) Dose-response recognition of peptide E75 by clones 3C4F (■) and 3B4E (○). Serial dilutions of peptide E75 were incubated with 3,000 T2 cells for 60 min. CTL were added at a E/T ratio of 4:1, and a standard 5-h cytotoxicity assay was performed. Lysis of T2 cells preincubated with E90 at 10 μM was <5% by both CTL clones. (*B* and *C*) Cold-target inhibition of lysis of freshly isolated ovarian tumor OVA-1 (*B*), and ovarian tumor clone SKOV3.A2.1E4 (*C*) by T2 cells preincubated with peptides E75 (■), E90 (□), and D132 (△). The effector (CTL-3C4F clone)/hot target ratio was 10:1. Peptide pulsed T2 cells (cold targets) were added in the assay at 5:1 and 10:1 cold/hot target ratios. Inhibition of lysis was determined in a 5-h ^{51}Cr release assay. Results are presented as percentages of inhibition of tumor target lysis by clone 3C4F, which was 46% for OVA-1 (*B*) and 28% for SKOV3.A2.1E4 (*C*). Lysis of parental control targets SKOV3 (HLA-A2$^-$) was 6% and of D132-pulsed T2 cells was 5% at the same E/T ratio. (*D*) Lysis of SKOV3.A2.1E4 was inhibited by anti–HLA class I (W6/32 mAb) but not by anti–HLA-DR (L243 mAb) at both 10:1 (▨) and 5:1 (■) E/T ratios.

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E/T ratios. These peptide concentrations are, for MAGE-3, gp100 and HER-2, significantly higher by at least two orders of magnitude than those of HLA-A2.1–restricted viral proteins (27).

To confirm that clone 3C4F recognizes a natural epitope associated with HLA-A2 on ovarian tumors, we examined the ability of E75 and E90 pulsed T2 cells to inhibit lysis of freshly isolated OVA-1 because CTL-1, autologous with this tumor, recognize only E75. Significant inhibition of lysis was observed by T2 cells pulsed with E75 but not with E90 (Fig. 3 *B*). Since antigen expression on freshly isolated ovarian tumors can be heterogeneous, to confirm that E75 represents an epitope presented by HLA-A2 on an ovarian tumor clone, the ability of E75 pulsed T2 cells to inhibit lysis of clone SKOV3.A2.1E4 was examined. Both OVA-1 and SKOV3.A2 transfectants share only HLA-A2 with effectors and express HER-2 on the surface, OVA-1: 79% HER-2$^+$ cells, MCF = 29; SKOV3 (positive control): 100% HER-2$^+$ cells MCF = 40; SKOV3.A2.1E4: 100% HER-2$^+$ cells, MCF = 40; C1R:A2 cells (negative control) MCF = 0.7. Ovarian clone SKOV3.A2.1E4 lysis by 3C4F clone was inhibited by anti–MHC class I (W6/32) but not by anti–MHC class II mAb (Fig. 3 *D*). Again, significant inhibition of lysis by clone 3C4F was observed in the presence of E75. Control peptide D132, which was not recognized when pulsed on T2 cells, failed to redirect clone 3C4F lysis (Fig. 3 *C*). Therefore, E75, which is recognized by four CTL lines and cloned CTL isolated from one of these lines, and which specifically inhibits recognition of ovarian tumors, may be a natural common HER-2 epitope recognized by ovarian-specific CTL.

Discussion

In this study, we have investigated recognition of synthetic peptide analogues of HER-2 epitopes containing HLA-A2–binding motifs by CD8$^+$CD4$^-$ CTL lines and clones isolated from TAL with ovarian tumors. We have identified one common epitope (E75) that is dominantly recognized by four out of four CTL lines. Of 19 peptides tested, another common epitope, C85, is recognized by two out of four lines. Several other epitopes, E89, E90, E71, and E73, are recognized only by one of the four CTL lines used, suggesting that they may be either private epitopes for these CTL or clones recognizing these epitopes are present with low frequency in the other CTL lines. The second possibility is more likely because the pattern of concentration-dependent recognition for E90 is similar with that of E75 and C85. In certain experiments, statistical analysis found that recognition of E89 and E90 by CTL-2 and CTL-4, and E91 by CTL-2 was significantly different from control targets, but the levels of recognition were lower (5–7%) than the cut-off value. We have observed that some ovarian CTL cultures lose the ability to recognize a number of these peptides over time probably because of gradual loss of lytic function or overgrowth of CTL of different specificities (Ioannides, C. G., and B. Fisk, unpublished data). We confirmed the specificity of E75 recognition by using two clones isolated from one of the CTL lines. E75 effectively inhibited lysis by CTL clones of both a freshly isolated ovarian tumor and an ovarian tumor line transfected with HLA-A2, indicating that the epitope recognized is not a culture artifact. Control peptides containing HLA-A2 anchor motifs (11, 12) but different intervening sequences failed to inhibit lysis, suggesting that a natural peptide with an identical or cross-reactive sequence is immunogenic in HLA-A2 ovarian cancer patients and may be presented on ovarian tumors.

Both E75 and C85 were recognized with different efficiencies by CTL1-4 at the same peptide concentration. This may be caused by the existence of clones in these CTL lines that recognize other as yet unknown antigens. The existence of multiple distinct ovarian Ag expressed simultaneously on the same tumor clone has been shown by analyzing recognition of ovarian clones by CTL isolated from TAL (1). Ovarian-specific CTL lines restricted by HLA-A2 recognize common epitopes present on allogeneic HLA-A2$^+$ ovarian tumors or lines, but not on HLA-A2$^+$ melanomas. Individual ovarian-specific CTL lines were found to recognize multiple Ag epitopes. Some of the common determinants may be expressed on other HLA-A2$^+$ epithelial tumors (2, 3). We have previously shown that ovarian TAL can recognize Muc-1 core peptides and HER-2, 968-984, a longer analogue of C85 peptide (HER-2, 971-979) (20). CTL-4 was isolated from TAL-24 used in these studies (20, 23). Recognition of Muc-1 by at least some of the clones derived from the CTL lines used in this study is likely. Although the percentage of tumor cells expressing Muc-1 in a tumor sample is variable and its expression is heterogenous, most ovarian tumors (>80% of serous adenocarcinomas) have been reported to express Muc-1 (23). Another HER-2 peptide, 654-662, derived from the transmembrane domain, was found to be recognized by TIL isolated from non–small cell lung cancer and developed by different methods (19). HER-2 is expressed in ∼30% of ovarian and breast carcinomas. However, its expression is relatively stable over time through the clinical course of invasive breast cancer, it is relatively congruent at all metastatic sites, and it is not affected by tumor heterogeneity (28). This has potential clinical applications because it may allow development of therapies based on HER-2 targeting (28).

Previous studies have shown a direct relationship between HER-2 overexpression and sensitivity to CTL of ovarian tumors (18). Since overexpression of HER-2 may induce expression of other proteins that can provide peptides (18) with the same or cross-reactive sequences, gene and protein sequence databases were searched for homologous sequences. 100% matches for both C85 and E75 were not found. For E75 only EGF-R (HER-1), HER-3, and HER-4 gave matches for the main HLA-A2 anchors at P2, P6, and P9, but nonconservative changes (underlined) were found in positions 1, 3, 5, and 7 (EGF-R, residues 364-372:SISGDLHIL, HER-3, residues 356-364:KILGNLDFL, HER-4:KINGNLIFL). Central positions are expected to be contact points for TCR (15, 29). Matches for peptide C85 appear in EGF-R, ERB.B3, DNA-directed RNA polymerase (RPB-1), and in an unknown nuclear protein (UL2-1). Nonconservative changes in the se-

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quence are dominant at positions expected to be TCR contacts such as P4 and P7 in RPB-1 and P5 in UL2-1. Based on recent crystallography data, the peptide termini are bound to HLA-A2 similarly but the central area of the peptide adopts different conformations that represent the epitopes recognized by TCR (29). Therefore, the nonconservative changes in the sequence of the homologous peptides from the other members of the HER family may affect epitope conformation and if these peptides are processed, presented, and recognized by TCR may constitute the equivalents of variants of peptides derived from the HER-2 protein.

As for the other tumor Ags (10, 25, 26), validation of HER-2 epitopes requires identification and quantitation of peptides bound to HLA class I on ovarian tumors. Since E75 lacks charged residues in the central area, it will be important to determine whether the same or conservatively substituted peptides from other proteins are naturally processed and presented to CTL. With the exception of E75 recognized by all four $CD8^+$ CTL, and in part of C85, which confirms our previous findings with unseparated CTL-TAL (20), three CTL recognized distinct HER-2 peptides at low level. These peptides were different in each system and their HLA-A2-stabilizing ability was variable over a wide range of concentrations. E89 binding affinity to HLA-A2 is at least three to four orders of magnitude lower than of naturally processed viral peptides (12), suggesting that the affinity of TCR for E89-HLA-A2 complexes may be high. This may also be true for C85. The affinity of a peptide for HLA-A2 is not the determining factor for the abundance of particular peptide presented by HLA-A2 (30). Other important factors are protein concentration and the processing efficiency of an antigenic peptide (30).

The mechanisms of HER-2 overexpression reflect gene amplification and upregulation of transcription (31). The involvement of translational, posttranslational mechanisms, or reduced rate of HER-2 turnover in HER-2 overexpression in cancer cells are still unclear (31). At this time, there is no simple explanation for the distinct pattern of peptide recognition between these lines. All tumors autologous with these CTL overexpressed HER-2 protein at similar levels that were consistent with HER-2 receptor overexpression when analyzed using monoclonal antibody *Ab2* that is specific for the extracellular domain of HER-2 (e.g., the levels of HER-2 expression were similar for OVA-1 and OVA-4 tumors [autologous with CTL-1 and CTL-4]: OVA-1, 79% $HER\text{-}2^+$ cells, MFC = 29, OVA-4, 100% $HER\text{-}2^+$ cells, MFC = 34, control OVA-17, 21% $HER\text{-}2^+$ cells, MFC = 4). However, CTL-4 associated with OVA-4 recognized in addition to E75 three other peptides. It is possible that these peptides if presented are processed by the tumor with different efficiencies (30). It is also possible that while for self-proteins the tolerance is not absolute, as shown for melanoma TIL specific for either MART-1 or gp100 (10, 25), elimination of high affinity T cells for a number of epitopes by tolerance may affect CTL with distinct specificities in each individual (10, 25).

Processing of overexpressed HER-2 in cancer cells may lead to peptides that differ in quantity from the HER-2 epitopes found on normal cells. Since HER-2 is present in normal epithelial tissues at lower levels and the protein concentration may be a limiting factor in epitope presentation, it will be important to determine how widely CTL-mediated HER-2 recognition is observed in ovarian and breast cancer patients, and whether such CTL can cause tumor rejection and show toxicity towards normal tissues.

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