Lysis of *Ras* Oncogene-transformed Cells by Specific Cytotoxic T Lymphocytes Elicited by Primary In Vitro Immunization with Mutated *Ras* Peptide

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Summary

Ras protooncogenes are activated by characteristic point mutations in a wide variety of malignancies. The expressed $p21^{ras}$ proteins are oncogenic by virtue of single substituted amino acids, usually at position 12 or 61 of the 189-residue $p21^{ras}$ protein. In the current study, the ability of class I major histocompatibility complex (MHC)–restricted T cells to recognize the altered segment of a transforming $p21^{ras}$ protein and to lyse cells transformed by the corresponding *ras* oncogene was examined. Synthetic *ras* peptides encompassing the common activating substitution of leucine for glutamine at position 61 were constructed with an amino acid motif appropriate for binding to the H-$2K^b$ murine class I MHC molecule. Cytotoxic T lymphocytes (CTL) specific for bound *ras* leucine 61 peptide were elicited by in vitro immunization of normal lymphocytes with synthetic peptides. The *ras* peptide–induced CTL specifically lysed syngeneic fibroblasts transformed by an activated *ras* gene encoding oncogenic $p21^{ras}$ protein containing the same single amino acid substitution. Thus, in some circumstances, mutated $p21^{ras}$ protein can serve as a tumor-specific antigen.

Tumor-specific CTL can eradicate antigenic tumors in experimental models (1). However, the use of specific CTL for cancer therapy has been limited by a paucity of defined tumor-specific antigens. In general, CTL recognize peptide fragments derived from endogenous cellular proteins, processed internally, and presented at the cell surface bound to class I MHC molecules (2–4). The appropriateness of any particular protein to serve as a target for CTL depends upon whether the protein has antigenic segments with the proper molecular configuration or amino acid "motif" to bind to class I MHC molecules; whether the resultant peptide–MHC molecular complex is presented at the cell surface in a concentration high enough to stimulate the antigen-specific TCR; and whether the peptide–MHC molecular complex is within the host T cell repertoire. Many tumor cells have been shown to harbor mutated protooncogenes with transforming activity. Theoretically, the abnormal proteins encoded by mutated protooncogene may contain peptide sequences as the result of activating mutations which are uniquely expressed in tumor cells and which potentially satisfy the necessary conditions for specific CTL recognition.

Mutated *ras* protooncogenes are particularly prevalent among human tumors (5). Activating point mutations of *ras* predominantly occur within codons 12 and 61 and result in the expression of a limited number of oncogenic $p21^{ras}$ proteins bearing single amino acid substitutions (5). The mutations disrupt the normal signaling function of $p21^{ras}$ and contribute to malignant transformation (6, 7). Oncogenic $p21^{ras}$ protein bearing a single amino acid substitution represents a tumor-specific protein present only in transformed cells. The current study elicited CTL specific for the mutated segment of a transforming $p21^{ras}$ protein by primary in vitro immunization with corresponding synthetic *ras* peptides and examined the ability of the mutated $p21^{ras}$ protein expressed by transformed cells to serve as a target for specific CTL lysis, i.e., to serve as a tumor-specific antigen.

Materials and Methods

Mice. Female C57Bl/6 (B6)[1] mice, 6–12-wk-old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the animal care facilities of the University of Washington.

[1] *Abbreviations used in this paper:* BLKSVHD, BLKSVHD.213; B6, C57Bl/6; CM, culture medium; D^b, H-$2D^b$; K^b, H-$2K^b$; T-OVA, trypsinized OVA.

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Tumor Cell Lines. BLKSVHD.213 (BLKSVHD), a SV40-transformed fibroblast line of B6 origin (8), was obtained from the American Type Culture Collection (Rockville, MD). FBL-3, a Friend virus–induced leukemia, and EL4, a chemically-induced thymoma, both of B6 origin, were subcloned and maintained in our laboratory (9). L cells transfected with H-2K^b (K^b) were a gift from Dr. S. Nathenson (Albert Einstein School of Medicine, New York) (10). The TAP-2–deficient cell line, RMA-S (derived from RBL-5 after mutagenization and selection with anti-H-2^b antiserum plus complement for loss of MHC class I expression), was a gift of Dr. C. Ohlen (Karolinska Institute, Stockholm, Sweden) (11). All cell lines were grown in culture medium (CM) consisting of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 2.5 × 10^{-5} M 2-ME, 200 U/ml penicillin, 200 U/ml streptomycin, 10 mM L-glutamine, and 10% FCS. Cells were kept in 25-ml flasks at 37°C, in a humidified atmosphere with 5% CO_2.

Peptides. Synthetic *ras* peptides were synthesized on a synthesizer (model 430A; Applied Biosystems, Foster City, CA) using BOC/benzyl based chemistry. Individual peptides were cleaved from the resin by reaction with hydrogen fluoride/anisole and purified by reversed phase chromatography. Characterization of the purified peptides was by amino acid analysis, analytical HPLC, and mass spectrometric determination of molecular weight. A mixture of 36 overlapping *ras* peptides was synthesized in a single multiplex procedure. The amino acid sequence of p21^{rasL61} from residue 53 through 69 is LDILDTAGLEEYSAMRD. The peptide mixture consisted of p53–61, –63, –64, –65, –66, –67, –68, –69; p54–61, –62, –63, –64, –65, –66, –67, –68, –69; p55–61, –62, –63, –64, –65, –66, –67, –68, –69; and p56–61, –62, –63, –64, –65, –66, –67, –68, –69. To construct the peptide mixture arginine (corresponding to residue 68 of p21^{ras}) was coupled to aspartyl (residue 69) resin, then arginyl resin was introduced into the reaction vessel and methionine (residue 67) was coupled to the mixture of the two resins. This strategy of alternate coupling of an amino acid followed by the addition of the same amino acid resin to the mixture was continued through L61. Residues G60, A59, T58, and D57 were coupled conventionally. Then residues L56, I55, D54, and L53 were successively coupled with resin being removed after each cycle. These four resulting resins with NH_2-terminal L56, I55, D54, and L53 were combined, cleaved with hydrogen fluoride, and the peptides were dissolved in aqueous acetic acid and chromatographed on Sephadex G-10. Synthetic OVA peptide p257–264 was a gift from Dr. S. Jameson (University of Washington, Seattle, WA). Peptide fragments of trypsinized OVA (T-OVA) were generated by tryptic digestion according to the method of Moore et al. (12).

Plasmids and Cell Transfection. The plasmid, pRSVL61, containing a synthetic *ras* gene which encodes p21$^{c\text{-}Ha\text{-}ras}$ with the single amino acid substitution of leucine for glutamine at residue 61 was kindly provided by Dr. S. Nishimura (National Cancer Center Institute, Tokyo, Japan) (13). The plasmid utilizes the LTR of Rous sarcoma virus to enable expression of the synthetic *ras* gene in eucaryotic cells. The Homer 6 plasmid containing the *aph* aminoglycoside phosphotransferase gene was obtained from Dr. O. Finn (Pittsburgh Cancer Institute, Pittsburgh, PA) (14). Plasmids were expanded in *Escherichia coli* strain HB101 and purified by CsCl centrifugation. BLKSVHD fibroblasts were cotransfected with pRSVL61 and Homer 6 using the calcium phosphate precipitation method of Sambrook et al. (15). Transformed cells were cloned and expanded in selection medium containing G418 at 1 mg/ml. The presence of the transfected synthetic *ras* gene was confirmed by PCR amplification and detection of the 567-bp synthetic *ras* gene, using the primers ATGACCGAATACAAACTG and AGACAGAACGCATTTGCA.

Peptide Binding Assay. The ability of synthetic *ras* peptides to bind to and stabilize K^b class I MHC molecules on the surface of RMA-S cells was measured by immunofluorescence. RMA-S cells were cultured for 2 h in the presence of CM alone or CM plus *ras* peptide (25 μg/ml). Cells were washed and stained with FITC-conjugated anti-K^b (Pharmigen, San Diego, CA). Labeled cells were again washed and analyzed for fluorescence on a FACStar® II (Becton Dickinson & Co., San Jose, CA). Fluorescence intensity was measured at 515–545 nm.

In Vitro Immunization Protocol. B6 spleen cells were immunized in vitro with the indicated peptide preparations according to previously described methods (16). Freshly harvested spleens were minced, washed, and suspended in CM at 5 × 10^6 cells per ml. A total of 5 × 10^7 spleen cells (vol. 10 ml) supplemented with sensitizing peptide (25 μg/ml) were incubated in upright 25 ml flasks for 7 d. The cultured cells were washed and tested in 4-h ^{51}Cr-release assays for the ability to lyse EL4 cells that had been preincubated for 2 h with designated targeting peptide (25 μg/ml).

Generation of T Cell Lines and Clones. T cell lines and clones were generated from in vitro–sensitized B6 spleen cells by repetitive stimulation of the primed spleen cells with synthetic *ras* peptides followed by rIL-2 as previously described (17). Primed spleen cells were transferred to 24-well plates at 4 × 10^6 cells per well with irradiated syngeneic spleen cells (3,000 rad) at 2 × 10^6 cells per well and the sensitizing peptide (25 μg/ml). The lymphocytes were cultured for 5 d then split at 1:2 into fresh CM. On day 10, lymphocytes were restimulated with Ag. 2 d later cultures were split 1:2 and supplemented with human rIL-2 (5 U/ml), generously provided by Hoffmann-La Roche, Inc. (Nutley, NJ). Thereafter, T cells were maintained by periodic restimulation with Ag (5 μg/ml) and irradiated syngeneic spleen cells, followed 2 d later by rIL-2 (5 U/ml). Cells were cloned by limiting dilution as previously described (17).

Cytotoxicity Assays. The cytotoxic activity of elicited T cells was measured by 4-h ^{51}Cr-release assays. Assays were performed as previously described (9). Where indicated, target cells were preincubated with the designated targeting or blocking peptides before use in ^{51}Cr-release assays.

Results and Discussion

Peptide binding with any particular MHC molecule is predicated on the component amino acids of the peptide being correctly aligned to the unique binding pockets of the MHC cleft (18). Key "anchoring" residues of antigenic peptides have been elucidated for a limited number of MHC molecules, including murine class I MHC molecules of the H-2^b haplotype (19). The region of p21^{ras} protein encompassing the residue 61 activation site contains a putative K^b-binding motif (19), consisting of Y64 and M67. One of the most common mutations of residue 61 consists of the substitution of leucine for glutamine. To determine whether, in fact, fragments of aberrant p21^{ras}, which contain an altered residue 61, can bind to class I MHC molecules—the sine qua non of CTL generation—synthetic peptides corresponding to the altered segment of p21^{rasL61} were assayed for binding and stabilization of K^b class I MHC molecules on the mutated cell line, RMA-S (H-2^b) (11).

Surface class I MHC molecules normally exist as trimolecular structures composed of class I H chain, bound peptide and β_2-microglobulin (20, 21). Both peptide and β_2-

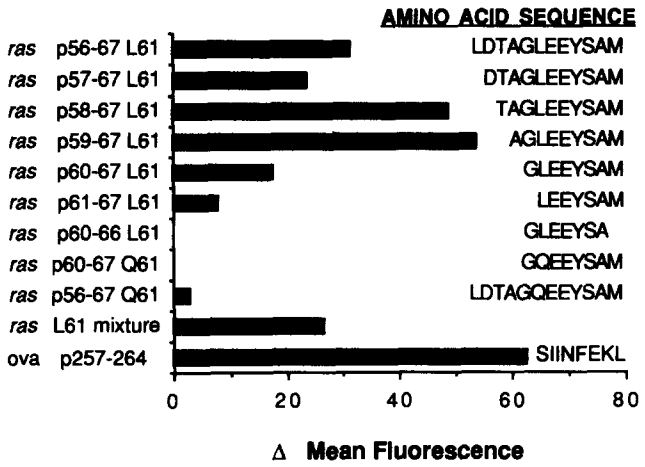

Figure 1. Stabilization of K^b expression on RMA-S by *ras* L61 peptides. RMA-S cells were cultured with the indicated peptide, then evaluated by immunofluorescent analysis for K^b expression as described in Materials and Methods. The data represents the difference between the mean fluorescence of cells cultured with the indicated peptide and the mean fluorescence of cells cultured without exogenously added peptide.

microglobulin are necessary for stable expression of class I MHC molecules at the cell surface. RMA-S cells express low levels of surface class I MHC molecules, attributable to the impaired transport of endogenously processed peptides for association with class I H chains (22, 23). However, provision of exogenous peptides that can bind to the unoccupied antigen binding groove, stabilize and enhance the expression of cell surface class I MHC molecules (24). Accordingly, RMA-S cells were incubated with a variety of synthetic peptides corresponding to the amino acid sequence of $p21^{rasL61}$ and assayed for expression of K^b (Fig. 1). *Ras* L61 peptides ranging from 7 to 12 residues in length and extending to M67 in their COOH terminus (p61–67, p60–67, p59–67, p58–67, p57–67, and p56–67) each stabilized expression of K^b on RMA-S to varying degrees. Maximal stabilization of K^b was observed with the 9-mer p59–67 peptide at levels comparable to that achieved with a known K^b-binding peptide, OVA p257–264 (25). The p60–66 *ras* L61 peptide lacking the putative M67 anchoring determinant, did not stabilize K^b expression. Moreover, normal *ras* Q61 peptide homologs containing the putative Y64 and M67 anchor residues did not stabilize K^b expression, indicating that the substitution of leucine for glutamine at residue 61 was important for the generation of a *ras* peptide segment capable of binding K^b. The L61 residue may act as a secondary anchor residue that binds to a corresponding subpocket of the K^b groove (18, 26, 27). Alternatively, the L61 residue may have conformational effects that enable the dominant anchor residues to align properly for binding to K^b.

The ability of K^b-binding *ras* L61 peptides to elicit CTL of the $H\text{-}2^b$ haplotype was examined. The generation of class I MHC–restricted CTL has classically required in vivo immunization with stimulator cells that synthesize the nominated target protein and that process and present the protein through the class I MHC pathway. Recently it has been shown to be possible to prime CTL in vivo by immunization with peptides that exhibit appropriate class I MHC binding motifs (28–30), thereby circumventing the need to immunize with viable cells synthesizing the nominated antigen. In a few instances, it has been possible to elicit CTL by priming with similar peptides in vitro, although CTL generated by peptide priming often fail to lyse cells that synthesize the parental target protein. In initial experiments, individual K^b-binding *ras* L61 peptides were tested for the ability to immunize B6 spleen cells in vitro. Multiple experiments to elicit CTL with individual K^b-binding *ras* L61 peptides ranging from 8 to 12 mer in size proved unsuccessful.

The generation of peptide-specific CTL response requires both peptide binding to class I MHC molecules and the presence of T cells with antigen-specific TCRs capable of recognizing that particular peptide–MHC complex. "Holes" in the T cell repertoire may exist for particular peptides bound in particular configurations. To circumvent the possibility that the *ras* L61 peptides used in initial experiments were not of the appropriate size or configuration for the induction of CTL, a panel of 36 overlapping *ras* L61 peptides was synthesized and used in combination to immunize lymphocytes in vitro. The peptides ranged from 6 to 17 residues in length and were systematically staggered from the NH_2 and COOH termini (see Materials and Methods). The presumption was that multiple CTL specific for several different peptide/K^b configurations would be elicited. Lymphocytes were cultured in vitro with the *ras* L61 peptide mixture and assayed for cytotoxicity. *Ras* L61 peptide–specific CTL were induced as evidenced by augmented lysis of syngeneic tumor cells (EL4) preincubated with the *ras* L61 peptide mixture compared to tumor cells alone (Table 1). The *ras* L61 peptide–specific CTL mediated enhanced lysis of tumor cells incubated with the sensitizing *ras* L61 peptide mixture but not with irrelevant peptide fragments of T-OVA. Conversely, CTL elicited by in vitro immunization to T-OVA peptides mediated enhanced lysis of tumor cells incubated with T-OVA peptides but not with the *ras* L61 peptide mixture (Table 1).

The specificity of the *ras* L61 peptide–induced CTL was further examined with individual *ras* L61 target peptides. A CTL line was established from in vitro–sensitized lymphocytes that expressed the $CD4^-CD8^+$ cell surface phenotype and that lysed EL4 tumor cells in the presence of selected *ras* L61 peptides (Table 2). Homologous peptides corresponding to normal $p21^{ras}$ protein, were unable to target lysis by the CTL line (Table 2). Moreover, irrelevant peptides with high affinity to K^b did not sensitize targets for lysis by the CTL line (data not shown). Thus, the *ras* L61 peptide–induced CTL specifically recognized only L61-substituted *ras* peptides.

The *ras* L61–specific CTL line strongly lysed targets pulsed with individual *ras* L61 peptides that stabilized K^b expression, but not with *ras* L61 peptides that did not stabilize K^b (Table 2). However, the degree of cytotoxicity directed against individual K^b-binding *ras* L61 peptide was not directly proportionate to the ability of the peptide to stabilize K^b on RMA-S. Smaller *ras* L61 peptides (7 and 8 mer), which were less effective than larger peptides in stabilizing K^b (Fig. 1),

Table 1. *Cytolytic Acitivity of Lymphocytes Immunized In Vitro with ras L61 Peptides*

Sensitizing peptide	Targeting peptide	Specific lysis 80:1	20:1	5:1
			%	
ras L61*	None‡	36§	15	3
ras L61	*ras* L61	50	26	8
ras L61	T-OVA	37	15	3
T-OVA	None	37	14	4
T-OVA	*ras* L61	37	12	4
T-OVA	T-OVA	52	29	8

* B6 spleen cells were incubated for 7 d with the indicated peptides at 25 μg/ml. *ras* L61 denotes the *ras* L61 peptide mixture and T-OVA denotes trypsinized OVA, described in Materials and Methods.
‡ ^{51}Cr-labeled EL4 cells were preincubated for 2 h with the indicated peptide at 25 μg/ml, then used as targets.
§ E/T ratio.

were more effective than larger peptides in sensitizing target cells for specific killing (Table 2). Most of the tested peptides which targeted specific killing were not intentionally constructed in the mixture of *ras* L61 peptides used for in vitro immunization. However, it is probable that such peptide fragments were present in the mixture as the result of partial synthesis and/or degradation (31).

The MHC restriction of the *ras* L61 peptide–induced CTL was established by chromium release assays with H-2 D^b and K^b transfected target cells. *Ras* L61–specific CTL lysed peptide-treated targets that exclusively expressed K^b (Table 3) but not targets that expressed D^b (data not shown). Coincubation of target cells with a synthetic OVA peptide (p257–264) with high affinity for K^b (25), markedly reduced the ability of *ras* L61 peptides to sensitize syngeneic target cells for lysis by *ras* L61-specific CTL (Fig. 2). By contrast, normal *ras* peptides, p56–67 Q61 and p60–67 Q61, did not block the ability of homologous *ras* L61 peptides to sensitize targets for lysis, providing additional evidence that normal *ras* Q61 peptides do not bind to K^b with high affinity.

Under normal circumstances, antigenic class I MHC–restricted peptide determinants are proteolytically excised from endogenously produced proteins (32). Potentially antigenic determinants nested within an intact protein may be destroyed by inappropriate cleavage of proteins by proteases during antigen processing. Furthermore, competing or suppressive determinants at other sites within a protein may mask CTL recognition of a potentially antigenic peptide segment (33). To establish whether endogenously expressed $p21^{rasL61}$ protein could be processed and presented to enable CTL recognition of the mutated segment of the protein, a fibroblast line of B6 origin (H-2^b) was transfected with the mammalian expression vector pRSV-L61 encoding $p21^{c\text{-}Ha\text{-}ras}$ protein with the activating L61 substitution (13) and used as a target in chromium release assays. The *ras* L61–specific CTL effectively lysed fibroblasts transformed by the mutated c-Ha-*ras* L61 gene, but not untransfected fibroblasts nor other syngeneic transformed cells (Fig. 3).

The current study demonstrates that peptide-specific CTL elicited by primary in vitro immunization can lyse transformed cells on the basis of the expression of an abnormal *ras* oncogene. The conditions developed to allow elicitation of peptide-specific CTL were derived by empiric experimentation. Large numbers of responder cells were required, presumably to compensate for a low frequency of CTL precursors (16). The reasons for the failure to demonstrate priming to individual *ras* L61 peptides, in light of the ability to prime to

Table 2. *Lysis of EL4 Tumor Cells Incubated with Various Synthetic ras Peptides by ras L61 Peptide–induced CTL*

Targeting *ras* peptide	Amino acid sequence	Specific lysis 40:1	20:1	10:1
	↓*		%	
ras L61‡		76§	70	62
p60–66 L61	G L E E Y S A	7	2	2
p61–67 L61	L E E Y S A M	81	76	67
p60–67 L61	G L E E Y S A M	72	59	55
p59–67 L61	A G L E E Y S A M	61	49	44
p56–67 L61	L D T A G L E E Y S A M	58	47	31
p60–67 Q61	G Q E E Y S A M	5	4	2

* The arrow denotes residue 61.
‡ ^{51}Cr-labeled EL4 cells were preincubated for 2 h with the indicated peptide (25 μg/ml) then used as targets.
§ E/T ratio.

Table 3. *Lysis of H-2K^b Transfected Cells Incubated with Various Synthetic ras Peptides by ras L61 Peptide-induced CTL*

Targeting *ras* peptide	Amino acid sequence	Specific lysis 40:1	20:1	10:1	5:1
	↓*		%		
ras L61‡		55§	49	45	37
p60–66 L61	G L E E Y S A	4	3	3	2
p61–67 L61	L E E Y S A M	56	51	50	47
p60–67 L61	G L E E Y S A M	49	39	30	26
p59–67 L61	A G L E E Y S A M	41	33	31	27
p56–67 L61	L D T A G L E E Y S A M	46	34	22	14
p60–67 Q61	G Q E E Y S A M	5	4	2	2

* The arrow denotes residue 61.
‡ ^{51}Cr-labeled L cells transfected with K^b were preincubated for 2 h with the indicated peptide (50 μg/ml), then used as targets.
§ E/T ratio.

the peptide mixture, have not been experimentally determined. However, it is most likely that the *ras* L61 peptide mixture concurrently primed CD4$^+$ T cells to provide requisite help for the elicitation of CD8$^+$ CTL responses. Previous studies have shown that longer *ras* L61 peptides are capable of eliciting Th cell responses (17), and many studies have emphasized the role of CD4$^+$ T cells in the generation of CTL (34).

In vivo immunization with similar *ras* peptides should engender similar CTL responses, with the caveat that little is known concerning the optimal methods to prime in vivo with class I MHC-binding peptides. CTL specific for the mutated region of p53 have been generated by in vivo priming (35). It might be possible to generate *ras*-specific CTL by the use of recombinant vaccinia virus encoding relevant *ras* proteins (36). However, antivaccinia responses may predominate, especially in previously primed individuals. Alternatively immunization with soluble protein has in some circumstances been reported to predispose to the generation of primed CTL to proteins, including p21^{ras} (37). However, the use of in vitro priming, especially if adaptable to humans, may allow a more precise determination of antigenic peptide epitopes and a more rapid determination of epitopes that are actually processed and presented by malignant cells. The further development of primary in vitro immunization may greatly hasten the realization of specific T cell therapy for malignancy.

The current study confirms that recognition of oncogenic *ras* proteins by mammalian CTL is possible. The ability of CTL to lyse transformed cells on the basis of the expression of an abnormal *ras* oncogene suggests that it might be feasible to define circumstances for eliciting and utilizing specific CTL

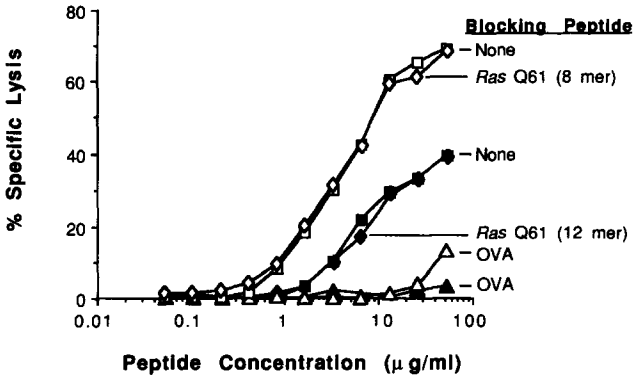

Figure 2. Inhibition of *ras* L61 peptide-specific cytotoxicity by the K^b-binding ovalbumin peptide, OVA p257–264. ^{51}Cr-labeled EL4 cells were incubated in serial dilutions of either *ras* p60–67 L61 (*open symbols*) or *ras* p56–67 L61 (*closed symbols*) peptides plus the indicated competing peptide (5 μg/ml) for 1 h, then used as targets in 4-h chromium release assays. Competing peptides included: *ras* p60–67 Q61 (8 mer), *ras* p56–67 Q61 (12 mer), and OVA p257–264. The data represent the percent specific lysis of the target cells by the *ras* L61-specific CTL line.

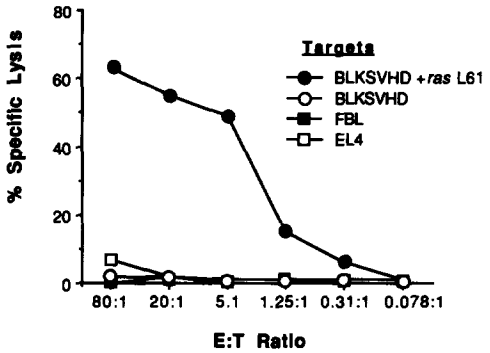

Figure 3. Lysis of p21^{rasL61}-transformed cells by syngeneic *ras* L61-specific CTL. The *ras* L61 peptide-induced CTL line was assayed for the ability to lyse the indicated target cells in 4-h chromium release assays. (BLKSVHD + *ras* L61) BLKSVHD cells transformed with the c-Ha-*ras* L61 oncogene.

to treat *ras* oncogene-transformed malignancies. The determination of whether *ras*-positive tumors elicit *ras*-specific immune responses and whether elicited immune responses affect tumor progression and/or the expression of $p21^{ras}$ protein may allow the elucidation of the concept of immune surveillance and/or immunological escape at a defined molecular level.

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