Brief Definitive Report

Identification of Two Cytotoxic T Lymphocyte-recognized Epitopes in the Ras Protein

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Summary

We have investigated the possibility of inducing cytotoxic T lymphocytes (CTL) to Ras containing a mutation at position 61 or to normal Ras, using recombinant vaccinia viruses expressing these proteins. CTL from C57Bl/10 mice immunized with vaccinia expressing mutant Ras showed specificity for the mutant Ras protein and recognition of normal Ras was inefficient. The opposite specificity was observed after immunization with vaccinia expressing normal Ras, since CTL isolated from these mice recognized normal Ras well and mutant Ras inefficiently. Levels of endogenous Ras expression were insufficient for lysis by these CTL. One CTL epitope mapped to amino acids 60–67 and residue 61 was critical for T cell recognition. CTL generated against mutant Ras protein recognized peptide 60–67 containing mutant residue 61, while anti–normal Ras CTL recognized the wild-type 60–67 sequence. A second epitope mapped to residues 152–159 of Ras and was recognized equally well by CTL raised to normal or mutant Ras. The murine data raise the possibility of exploiting Ras-specific CTL for targeted immunotherapy of certain human cancers.

The concept of immunotherapy of cancer rests on the activation of effector mechanisms that can specifically recognize and eliminate tumor cells. Since CTL are responsible for tumor rejection in many experimental models (1), they represent promising anticancer effectors. The limited success of reintroducing cytokine-activated lymphocytes into cancer patients might be due to the lack of specificity (2, 3). Although it is possible to isolate CTL from tumor-infiltrating lymphocytes that specifically lyse the patients tumor cells (4–7), the antigens recognized are usually unknown, making targeted therapy a difficult task. Significant progress was made recently when the gene encoding a CTL-recognized melanoma antigen was cloned and shown to be expressed in 40% of human melanomas (8).

Oncogenes are attractive targets for immune intervention because they are often mutated or overexpressed in cancer cells (9). The Ras protein is implicated in a large number of human malignancies, contributing to transformation by abnormally high levels of expression (10–12) or through mutations at either position 12, 13, or 61 (13–15). Although MHC class II–restricted Th cells have been generated to synthetic Ras peptides (16–19), it remained unclear whether these T cells can recognize cells expressing Ras endogenously. Since Ras is not a secreted or cell surface protein and not known to be present in the endo-lysosomal compartment where loading of MHC class II molecules normally occurs (20), presentation to Th cells might be very inefficient. In contrast, the cytoplasmic location of Ras may allow ready access to the MHC class I presentation pathway (21), which is required for CTL recognition. In this study we have tested whether peptide epitopes derived from endogenously expressed Ras are presented by class I molecules and whether they stimulate CTL responses.

Materials and Methods

Cell Lines. RMA is a Rauscher virus–induced T cell lymphoma of C57BL/6 origin, and RMA-S is a mutagenized, anti-H-2-selected subline of RMA (22). EL4 is a mutagen-induced T cell lymphoma isolated from C57BL/6 mice (23). P1HTR is a subline of the P815 mastocytoma of DBA/2 origin selected for high transfection frequency (24). P1HTR cells were transfected with a genomic clone of H-2K^b, and P1HTR-D^b transfectants were a gift from E. Simpson (Clinical Research Center, Harrow, London, UK). EL4 transfectants were generated by electroporation with the retroviral vector pBabe-Hygro (gift from Hartmut Land, Imperial Cancer Research Fund, Lincoln's Inn Field, London, UK) containing cDNAs encoding normal or 61 mutant human N-Ras (see below) under the promoter control of moloney murine leukemia virus LTR. After electroporation, hygromycin-resistant clones were isolated and tested by Southern blotting for the presence of intact N-*ras* genes. Densitometer analysis indicated that the normal N-Ras– and the mutant N-Ras–transfected clones used in this study contained approximately five gene copies. All the cell lines were maintained in RPMI 1640 plus 10% FCS.

Recombinant Vaccinia Virus. cDNAs encoding human N-Ras or

A Anti-Ras61 CTL

B Anti-Ras CTL

C Anti-Ras61 CTL

D Anti-Ras CTL

E Anti-Ras61 CTL
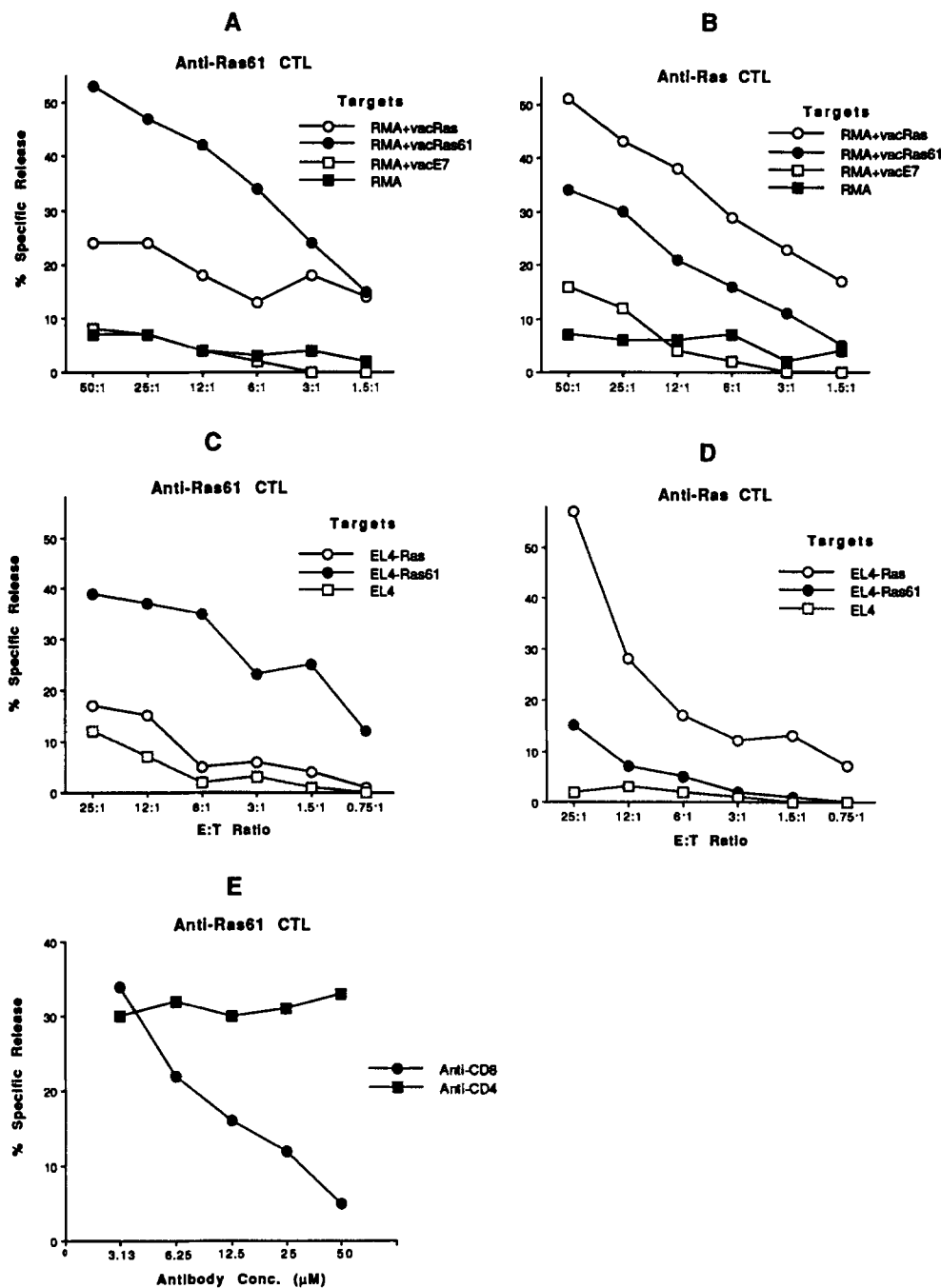
Figure 1. CD8+ CTL lyse Ras-expressing target cells. Ras-specific CTL were isolated from C57Bl/10 mice immunized with Vac-Ras[61] or with Vac-Ras. Target cells are RMA (H-2b) infected with the indicated vaccinia viruses (A and B), or EL4 (H-2b) transfected with Ras[61] or normal Ras (C and D). E shows lysis of RMA cells infected with Vac-Ras[61] in the presence of mAb to CD8 (YTS 169.4) or CD4 (GK1.5). CTL activity in the presence of CD4 antibodies was the same as without antibodies (not shown).

N-Ras with a glutamine to lysine mutation at position 61 (gifts from Alan Hall, Institute of Cancer Research, Chester Beatty, London, UK) were inserted into the vaccinia expression plasmid pSCII, and recombinant virus was generated as described (25). Recombinant virus containing the E7 gene of human papilloma virus (Vac-E7; gift from Lionel Crawford, Department of Pathology, Cambridge University, Cambridge, UK) was used as control in CTL assays.

CTL Generation. 2 × 10^{7} PFU of recombinant virus Vac-Ras or Vac-Ras[61] was used to immunize C57Bl/10 mice (obtained from the ICRF breeding colony at Clare Hall, London, UK) by intraperitoneal injection. Mice were boosted with the same virus dose

2 and 3 wk later. 10 d after the last boost splenocytes were restimulated for 5 d in 10-ml cultures containing 5 × 10^{7} responder spleen cells and 10[6] stimulator RMA cells infected with Vac-Ras or Vac-Ras[61], respectively. CTL lines were established by weekly stimulation of responding T cells in 2-ml cultures containing 8 × 10^{5} T cells, 10[5] infected RMA stimulators, and 2 × 10^{6} irradiated (3,000 rad) syngeneic feeder splenocytes.

CTL Assays. CTL activity was tested in standard 4-h 51Cr release assays using 51Cr-labeled target cells as described (26). Briefly, RMA or EL4 cells were 51Cr labeled for 1 h at 37°C and then seeded at 5 × 10^{3} per well in 96-well microtiter plates. Effector CTL were added to obtain indicated E/T ratios, and after a 4-h

incubation at 37°C, 100 out of 200 μl medium per well was harvested and radioactivity was counted in a gamma counter (Pharmacia/LKB). Percent specific lysis was calculated using the equation: 100× [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. RMA target cells were infected with Vac-Ras, Vac-Ras[61], or Vac-E7 (10 PFU/cell) for 3 h at 37°C before labeling with ^{51}Cr. In antibody inhibition experiments, anti-CD8 and anti-CD4 mAbs were present during the 4-h CTL assay at the indicated concentrations.

Antibodies. mAbs Y3 (27) (anti-K^b) and B22 (28) (anti D^b) were gifts from Dr. A. Townsend (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK). For FACS® staining (Becton Dickinson & Co., Mountain View, CA), undiluted culture supernatants were used. mAbs to CD8 (YTS 169.4 [29]) or CD4 (GK1.5 [30]) were used for CTL blocking experiments.

Peptides. Ras peptides P60^N (GQEEYSAM) P60^M (GKEEYSAM) correspond to amino acids 60–67 of normal or mutant N-Ras, respectively, and P152 (VEDAFYTL) represents amino acids 152–159. Peptide nucleoprotein (NP) 345–360 corresponds to a K^b-binding epitope present in the nuclear protein of influenza virus, and peptide NP366–374 is a D^b-binding epitope (31). Peptide E-7 20–28 corresponds to a K^b-binding epitope in the E7 protein of human papilloma virus (32). All peptides were synthesized by the ICRF Central Laboratory Services with free NH$_2$- and COOH-terminal ends using solid phase Fmoc chemistry, and their purity was analyzed by HPLC and mass spectrometry.

MHC Class I Binding Assay. RMA-S cells were cultured at 26°C overnight to upregulate levels of class I expression (33). Cells were seeded in triplicates on 96-well plates (2 × 10^5 cells/well), and Ras peptides or K^b or D^b binding control peptides were added. After incubation for 1 h at 37°C, levels of class I expression were determined by indirect immunofluorescence staining and FACS® analysis as described (32).

Results and Discussion

Since vaccinia virus has been shown to be efficient in inducing CTL responses (34–36), we have chosen to use this vector for stimulation of anti-Ras responses in C57Bl/10 mice. DNAs encoding normal human N-Ras or mutant N-Ras containing a glutamine to lysine change at position 61 were inserted into the vaccinia genome and expressed from the early-late 7.5 promoter. Mice were immunized and boosted twice and spleen cells were restimulated in vitro with infected stimulator cells.

CTL lines from animals immunized with Vac-Ras[61] lysed H-2^b-derived RMA cells infected with the immunizing virus more efficiently than Vac-Ras-infected cells (Fig. 1 *A*), while CTL from Vac-Ras-immune mice lysed targets expressing Ras better than Ras[61]-expressing cells (Fig. 1 *B*). Target cells infected with a control vaccinia construct expressing the E7 protein of human papilloma virus showed only background lysis, indicating that vaccinia-encoded proteins did not stimulate detectable CTL responses. The observed Ras-specific lysis was mediated by CD8$^+$ T cells since anti-CD8 but not anti-CD4 mAbs inhibited lysis (Fig. 1 *E*). CTL lysis was not dependent upon vaccinia infection but was also seen with transfected target cells. Ras[61]-specific CTL lysed EL4 cells transfected with Ras[61] well and showed little lysis of target cells transfected with normal Ras (Fig. 1 *C*). Conversely, Ras but not Ras[61] transfectants were lysed
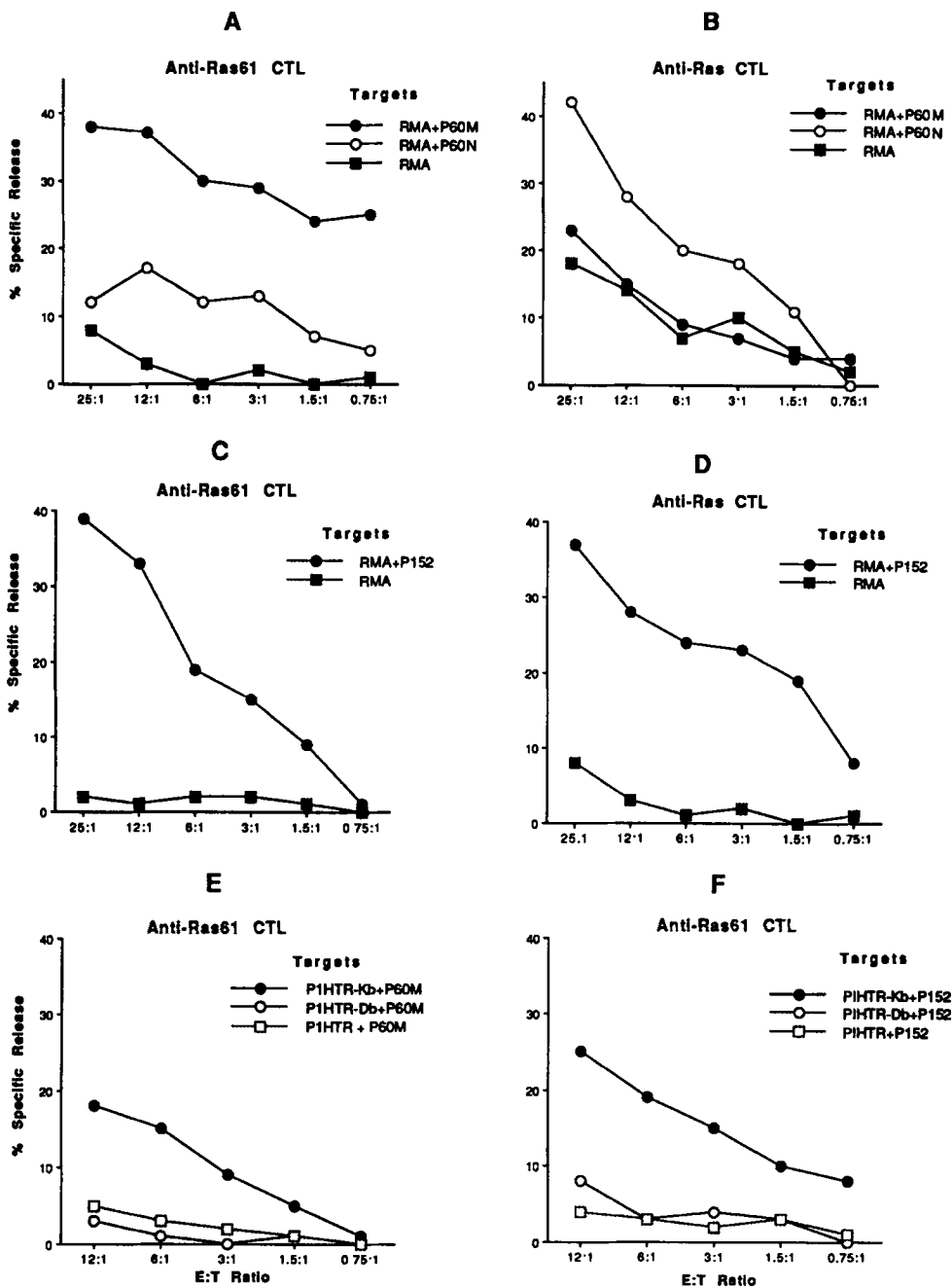
efficiently by CTL raised against normal Ras (Fig. 1 *D*). These CTL did not lyse untransfected EL4 cells, indicating that levels of endogenous Ras expression were insufficient for CTL recognition.

To map the epitopes recognized by Ras-specific CTL lines, we took advantage of known peptide binding motifs for the MHC class I molecules K^b and D^b (37). Two K^b but no D^b motifs were present in the Ras sequence. Amino acids 60–67 contained a K^b motif and we synthesized 8mer peptides P60^N and P60^M corresponding to the normal or mutant Ras sequence, respectively. Peptide P152, containing the second K^b motif spanning amino acids 152–159, was also synthesized. First, binding to class I molecules was measured in a cell surface binding assay (32) using the peptide loading-deficient cell line RMA-S. All three Ras peptides bound to K^b but not D^b (Fig. 2), demonstrating the value of predictive binding motifs. CTL generated against Ras[61] lysed target cells pulsed with P60^M well and showed some cross-recognition of peptide P60^N (Fig. 3 *A*), while CTL raised against normal Ras recognized P60^N and showed little cross-recognition of P60^M (Fig. 3 *B*). Both CTL recognized target cells pulsed with peptide P152 equally well (Fig. 3, *C* and *D*). As expected, CTL recognition of P60 and P152 was K^b restricted, which was confirmed by lysis of K^b- but not D^b- transfected H-2^d target cells (Fig. 3, *E* and *F*). The responses of C57Bl/10 mice to these epitopes were not due to species differences of human and mouse N-Ras, since they are identical except for three COOH-terminal residues, 168, 184, and 188 (38).

Together, the data show that C57Bl/10 mice can generate CTL responses to Ras and that at least two K^b-restricted
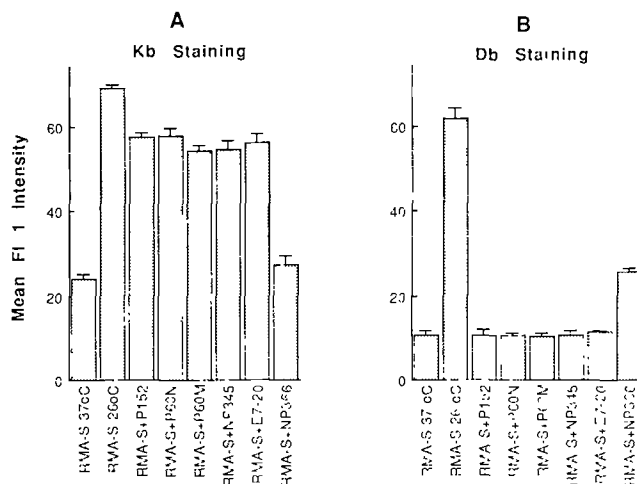
Figure 2. MHC class I binding of Ras peptides. Ras peptides P60N, P60M, and P152 stabilize K^b (*A*) but not D^b (*B*) class I molecules in temperature-induced RMA-S cells. For comparison, the stabilizing effects of known K^b-binding peptides NP345 (31) and E7-20 (32) are shown. NP366 (31) served as positive control for D^b binding. All peptides were used at a concentration of 50 μM. In the absence of class I-binding peptides expression of temperature-induced molecules (26°C samples) decreased significantly (37°C samples). All samples were done in triplicate and standard deviations are shown.

A

Anti-Ras61 CTL

Targets
●—— RMA+P60M
○—— RMA+P60N
■—— RMA

% Specific Release

B

Anti-Ras CTL

Targets
●—— RMA+P60M
○—— RMA+P60N
■—— RMA

C

Anti-Ras61 CTL

Targets
●—— RMA+P152
■—— RMA

% Specific Release

D

Anti-Ras CTL

Targets
●—— RMA+P152
■—— RMA

E

Anti-Ras61 CTL

Targets
●—— P1HTR-Kb+P60M
○—— P1HTR-Db+P60M
□—— P1HTR + P60M

% Specific Release

E:T Ratio

F

Anti-Ras61 CTL

Targets
●—— P1HTR-Kb+P152
○—— P1HTR-Db+P152
□—— P1HTR+P152

E:T Ratio

Figure 3. Recognition of Ras peptides P60^N, P60^M, and P152 by anti-Ras CTL. ^{51}Cr-labeled RMA cells (H-2^b) or P1HTR cells (H-2^d) transfected with K^b or D^b were incubated for 1 h at 37°C with 50 μM of the indicated peptides and then used as targets in a 4-h ^{51}Cr release assay. CTL were from C57Bl/10 mice immunized with Vac-Ras[61] or with Vac-Ras.

epitopes were recognized. This indicates that endogenous Ras does not lead to clonal deletion of Ras-specific T cells. One possibility is that physiologic levels of Ras expression are immunologically silent because they are insufficient for T cell recognition. This is supported by the observation that effector CTL only recognized Ras-transfected or Vac-Ras-infected targets, but not unmanipulated cells. We are not aware of a H-2^b tumor cell line overexpressing spontaneously mutant or normal Ras, to test whether transforming levels of Ras expression would lead to CTL lysis. In vivo tumor challenge experiments with Ras transfectants will reveal whether anti-

Ras CTL can mediate tumor protection in mice. The selective lysis of cells expressing high levels of Ras might allow the design of a CTL-based immunotherapy of certain human tumors. Recognition of transformation-associated Ras mutations would confer strict tumor specificity to CTL. Although the recognition of a mutation-containing epitope in C57Bl/10 mice is very promising, in an outbred human population the CTL-recognized epitopes will clearly depend upon the MHC haplotype. Nevertheless, the results obtained in the murine system encourage the search for Ras epitopes recognized by human CTL.

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