Advances in Brief

Generation of Anti-p53 Cytotoxic T Lymphocytes from Human Peripheral Blood Using Autologous Dendritic Cells[1]

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Abstract

CTLs recognizing the HLA-A2.1-restricted, wild-type sequence p53 epitopes $p53_{149–157}$ and $p53_{264–272}$ were generated from CD8-enriched populations of nonadherent peripheral blood lymphocytes (PBLs) obtained from healthy donors. The PBLs were restimulated *in vitro* with peptide-pulsed granulocyte macrophage colony-stimulating factor- and interleukin (IL)-4-induced autologous dendritic cells in the presence of IL-6 and IL-12 and subsequently cultivated with IL-1α, IL-2, IL-4, IL-6, and IL-7. Bulk anti-$p53_{264–272}$ CTL populations were generated from PBLs obtained from two of five donors. Both CTL populations were cytotoxic against peptide-pulsed HLA-$A2^+$ target cells, but not against untreated target cells. A $CD8^+$ anti-p53 CTL clone designated p264#2 was isolated from one of the bulk populations. It was found to have an intermediate affinity of approximately 10^{-9} M for the epitope and to mediate cytotoxicity against several human tumor cell lines, including the squamous cell carcinoma of the head and neck cell line SCC-9, which is known to present the wild-type sequence $p53_{264–272}$ epitope. In addition, CTLs reactive against $p53_{149–157}$-pulsed targets as well as a HLA-$A2^+$ tumor cell line were cloned from a bulk population of antitumor CTLs obtained from one of the five normal PBLs restimulated with this epitope. The results indicate that CTLs recognizing wild-type sequence epitopes can be generated from precursors present in PBLs obtained from some normal individuals using autologous dendritic cells as antigen-presenting cells and suggest that vaccine strategies targeting these epitopes can lead to antitumor CTL generation, thereby emphasizing the therapeutic potential of p53-based cancer vaccines.

Introduction

The rapid progress made in the identification of CTL-defined melanoma antigens and in their introduction into the clinic as components of cancer vaccines has increased interest in the identification of CTL-defined tumor antigens expressed by a broad variety of human tumor types (1). The *p53* gene has been shown to be frequently mutated in a wide range of human cancers (2) and has long been viewed as an ideal target for therapeutic interventions ranging from replacement gene therapies to cancer vaccines and immunotherapy (3–7). Missense mutations in p53 could be targeted for tumor-specific immunization, with the caveat that these mutations occur within peptides capable of being processed and presented by MHC molecules expressed by the host (8). Even if this requirement is fulfilled, however, vaccines based on mutated p53 epitopes are limited to individual patients. However, alterations in *p53* as well as in other genes can frequently result in the accumulation (overexpression) of mutated and/or wild-type p53 molecules in tumors (9). Thus, enhanced presentation of wild-type sequence epitopes derived from p53 molecules accumulating in tumors is possible and might lead to recognition by the immune system and the development of antitumor CTLs. This situation is comparable to recognition by antimelanoma CTLs of wild-type sequence epitopes derived from nonmutated gene products overexpressed in melanomas (10, 11). Clearly, cancer vaccines targeting the induction of CTLs recognizing such determinants would have broader applicability than a custom-made vaccine targeting an idiotypic, mutated p53 epitope.

It has been known for years that p53 serves as a target for immune recognition, with as many as 20% of patients having circulating antibody to p53 in a variety of malignancies, including breast, colorectal, and head and neck carcinomas and hepatoma (12, 13). In fact, p53 was initially identified as a tumor antigen by DeLeo *et al.* (14). More recently, we focused on p53 as a tumor antigen and identified p53 peptide epitopes suitable for presentation by HLA-A2 (6, 15, 16). We have also demonstrated in a murine model that immunization to the wild-type sequence $p53_{232–240}$ epitope resulted in the development of CTLs recognizing this epitope, which were cross-reactive against murine tumors expressing p53 molecules with mutations outside of this epitope (17).

Presently, two wild-type sequence human p53 peptides, $p53_{149–157}$ and $p53_{264–272}$, have been identified as HLA-A2.1-restricted CTL-defined epitopes by lymphocytes obtained from either healthy HLA-$A2^+$ individuals or HLA-A2-transgenic mice immunized with human p53 (18–22). With the exception of the CTLs generated in HLA-A2-transgenic mice deficient for p53 (21), anti-p53 CTL effectors do not appear to have high

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affinities for their ligand and tend to prefer to lyse target cells pulsed with p53 peptides, but not tumor cells accumulating p53.

Induction of CTLs is considered to be optimal when professional APCs,[3] such as DCs, process and present an antigenic epitope to T cells (23, 24). In several murine tumor antigen systems, the use of DCs as APCs has resulted in the induction of potent antigen-specific CTLs (17, 25–28). In our own studies, immunization of mice with DC-pulsed with mutant or wild-type sequence $p53_{232–240}$ peptide induced relatively potent anti-p53 CTLs, which proved to be effective in mediating tumor rejection in the prophylactic and therapeutic settings (17). The observed antitumor effects occurred in the absence of any noticeable, deleterious auto-immune anti-self effects that might theoretically take place as a result of CTL reactivity against normal tissue expressing wild-type p53 epitopes. These observations support the further development of safe p53-based immunotherapy (29).

In the current report, we have analyzed the *in vitro* induction of CTLs recognizing the HLA-A2.1-associated wild-type sequence $p53_{149–157}$ and $p53_{264–272}$ epitopes by restimulation of CD8-enriched lymphocytes obtained from healthy HLA-$A2.1^+$ donors, using peptide-pulsed autologous DCs as APCs. The bulk CTL populations as well as CTL clones isolated from the bulk cultures were analyzed for their reactivities against peptide-pulsed HLA-$A2^+$ target cells and HLA-$A2^+$ tumor cell lines that naturally present these wild-type sequence p53 epitopes.

Materials and Methods

Cell Lines. The tumor cell lines used in this study and their phenotype with respect to the expression of HLA-A2 and p53 are listed in Table 1. The SCCHN cell lines used in this study, PCI-4B, PCI-13, PCI-23, and PCI-30, and a gastric carcinoma cell line, HR, have been described previously (30). SCCHN cell line OSC-19 was a kind gift from Dr. E. Yamamoto (Department of Maxillofacial Surgery, Kanazawa University, School of Medicine, Kanazawa, Japan). SCCHN cell lines SCC-4 and SCC-9, the p53-null osteosarcoma cell line SaOS-2, and the breast cancer cell line MCF-7 were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum. The transporter associated with antigen processing (TAP)-deficient (TxB) cell hybrid T2 cell line was maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum (Life Technologies, Inc.), as were chronic myelogenous leukemia cell line K562 and a HLA-$A2^+$ EBV-transformed B-cell line (EBV-B) established in our laboratory and used in this study as a feeder cell for cloning T cells. The $p53^+$ cell line SaOS-2cl3 was derived by the transduction of p53-null SaOS-2 cells with p53 cDNA expressing a p53 missense mutation in codon 148 (2, 20).

[3] The abbreviations used are: APC, antigen-presenting cell; DC, dendritic cell; SCCHN, squamous cell carcinoma of the head and neck; PBL, peripheral blood lymphocyte; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody.

Table 1 Normal cells and tumor cell lines used in this study

Cells	HLA-A2 expression[a]	p53 status[b]	$p53_{264–272}$ presentation
Tumors			
HR	+	mm/codon 306[c]	ND
MCF-7	+	wt[d]	+
SCC-4	+	mm/codon 155[e]	−
SCC-9	+	Deletion 275–285[e]	+
OSC-19	+	mm/codon 225	ND
PCI-4B	−	wt	−
PCI-13	+	mm/codon 286	+
PCI-30	+	wt	ND
Normal cells			
PHA-lymphoblasts	+	wt	−

[a] HLA-A2 expression was determined serologically.
[b] Based on sequence analysis of reverse transcription-PCR products corresponding to p53 exons 5–8, as detailed in "Materials and Methods" or in the cited references.
[c] mm, missense mutation; wt, wild-type sequence; ND, not determined.
[d] Based on the analysis detailed in Ref. 20.
[e] Based on the analysis detailed in Ref. 19.

The transfectants were selected by growth in the presence of G418, cloned, and analyzed for p53 expression by immunoblot analysis using a rabbit antiserum raised in our laboratory against a synthetic peptide corresponding to murine $p53_{220–235}$, which was conjugated to keyhole limpet hemocyanin.

p53 Mutation Analysis. Sequence analyses of *p53* exons 5–8 expressed in the PCI-4B, PCI-13, PCI-30, OSC-19, and HR cell lines were performed by Dr. John C. Law (Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA; Ref.31) and are summarized in Table 1. PCI-13 was found to express a p53 missense mutation in codon 286 (Glu to Lys), OSC-19 expressed a mutation in exon 5 of codon 164 that generates a termination codon (AAG→TAG), whereas PCI-4B and HR were wild-type with respect to exons 5–8. The p53 mutation analysis of SCC-4 and SCC-9 has been described previously (19). The SCC-4 cell line expresses p53 with a missense mutation in codon 151 and accumulates p53, whereas SCC-9 expresses p53 molecules in which codons 285–295 have been deleted, and it does not accumulate p53 molecules.

Peptides. The HLA-A2.1-binding peptides STPPPGTRV and LLGRNSFEV, corresponding to $p53_{149–157}$ and $p53_{264–272}$, were synthesized using standard *N*-(9-fluorenyl)methoxycarbonyl methodology, purified, and stored as lyophilized preparations. Their amino acid sequences were confirmed by mass spectroscopy. The peptides were dissolved in DMSO at 1 mg/ml and diluted with PBS just before use.

Cytokines. The cytokines used in this study were obtained from the following sources: (*a*) human recombinant IL-1α, Genzyme (Cambridge, MA); (*b*) human recombinant IL-2, Chiron-Cetus (Emeryville, CA); (*c*) GM-CSF and IL-4, Schering-Plow (Kennilworth, NJ); and (*d*) IL-6, Sandoz (Basel, Switzerland). IL-7 and IL-12 were provided by M. T. Lotze (University of Pittsburgh, Pittsburgh, PA), whereas IFN-γ and TNF-α were kindly provided by Roussel UCLAF (Romainville, France) and Knoll Pharmaceuticals (Whippany, NJ), respectively.

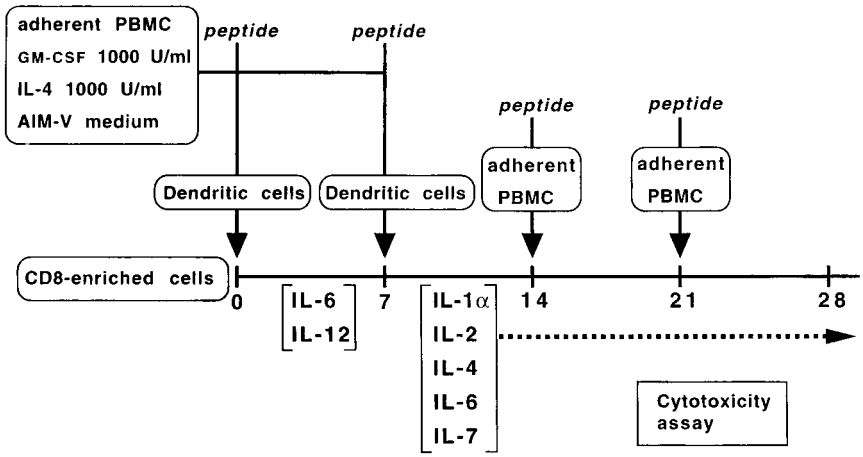

Fig. 1 The scheme for induction of anti-p53 CTLs from PBLs of healthy donors.

***In Vitro* Induction of Anti-p53 CTLs Using Peptide-pulsed Autologous DCs.** Peptide-specific CTL lines were generated as follows (Fig. 1): PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation of venous blood obtained from HLA-A2.1$^+$ healthy donors. DCs were generated from PBMCs using a modification of the method reported by Sallusto and Lanzavecchia (32). PBMCs were resuspended at a concentration of 5–10 $\times$ 10^6 cells/ml in AIM-V medium (Life Technologies, Inc.) and placed in T-162 flasks (Costar, Cambridge, MA). After a 2-h incubation at 37°C, the nonadherent cells were removed by gentle washing, and AIM-V medium containing GM-CSF and IL-4 (1000 units/ml each) was added. After 6 days of incubation, IL-1α (50 units/ml) was added to the medium. One day later, nonadherent DCs were harvested and used as APCs. The DCs were resuspended at a concentration of 2 $\times$ 10^6 cells/ml in AIM-V medium containing 40 μg/ml peptide and 3 μg/ml human β_2-microglobulin (Sigma Chemical Co., St. Louis, MO) and incubated at 37°C for 4 h. Subsequently, the peptide-pulsed DCs were irradiated (3000 rads), centrifuged, and resuspended in AIM-V medium containing 5% (v/v) human AB serum. Autologous CD8-enriched T cells were prepared by the depletion of CD4$^+$ T cells from lymphocytes using anti-CD4 mAb (DAKO, Carpinteria, CA) and goat anti-mouse IgG-coated magnetic beads (Advanced Magnetics, Cambridge, MA). On day 0, 3 $\times$ 10^6 responder cells and 3 $\times$ 10^5 peptide-loaded DCs/well were cocultured in the wells of a 24-well tissue culture plate (Costar) in a final volume of 2 ml/well AIM-V medium supplemented with 5% (v/v) human AB serum, 1000 units/ml IL-6, and 5 ng/ml IL-12. On day 7, the responder cells were restimulated with peptide-pulsed autologous DCs (day 14) in AIM-V medium supplemented with IL-1α (10 units/ml), IL-2 (5 IU/ml), IL-4 (50 units/ml), IL-6 (125 units/ml), and IL-7 (30 units/ml). On day 14 and weekly thereafter, the responder cells were restimulated with peptide-pulsed autologous PBMCs in the presence of the cytokine mixture. These cells were prepared as follows: cryopreserved PBMCs were thawed and irradiated; and 4 $\times$ 10^6 cells/ml AIM-V/well were incubated for 2 h in the wells of a 24-well plate. Nonadherent cells were removed, and a 0.5-ml aliquot of AIM-V medium containing 10 μg/ml peptide and 2 μg/ml β_2-microglobulin was added to each well. After a 2-h incubation, the peptide-containing medium was removed, and the responder lymphocytes were added in cytokine-supplemented medium. Responder cells were tested for their specificity after two or more rounds of restimulation.

The CTLs were cloned from the bulk cell lines by limiting dilution. Cells were plated at a density of 1 cell/well in the wells of 96-well plates. Each well contained 1 $\times$ 10^5 irradiated (3,000 rads) PBMCs from two different donors in 0.2 ml of AIM-V medium containing 5% (v/v) AB serum, IL-2 (100 IU/ml), IL-4 (50 units/ml), and anti-CD3 mAb (OKT-3; American Type Culture Collection; 2 mg/ml) per well. Peptide-specific CTL clones were then restimulated and expanded using irradiated (10,000 rads) allogeneic A2$^+$ EBV-B cells pulsed with the peptide as stimulators and feeder cells.

Chromium Release Assay. Standard 4-h ^{51}Cr release assays were performed as described previously (30). Briefly, 10^6 target cells were incubated with 100 mCi of $Na_2{}^{51}CrO_4$ for 1 h, washed, and dispensed into the wells of V-bottomed 96-well microtiter plates. Monolayer tumor targets cells used in this study were pretreated overnight with IFN-γ (1000 IU/ml) before ^{51}Cr labeling. Effector cells (0.2 ml) were added to 1 $\times$ 10^3 target cells in triplicate wells. Some target cells were preincubated with peptide for 1 h before the addition of effector cells. In antibody blocking experiments, hybridoma supernatants were added at a final dilution of 1:10. After a 4-h incubation at 37°C, the supernatants were harvested, and ^{51}Cr release was measured in a scintillation counter.

Cytokine Release Assays. CTLs (1 $\times$ 10^4) were incubated with 1 $\times$ 10^4 stimulator cells in 0.2 ml of AIM-V medium containing 5% human AB serum without cytokines. After 24 h, the supernatant was collected, and the GM-CSF and TNF-α content was determined by ELISA. The ELISAs for GM-CSF (Endogen, Inc., Woburn, MA; sensitivity, 5 pg/ml) and TNF-α (Biosource, Int., Camarillo, CA; sensitivity, 5 pg/ml) were performed at the Immunological Monitoring Laboratory, University of Pittsburgh Cancer Institute. The ELISA kits were calibrated against WHO standards for the two cytokines, and the coefficient of variation was 11% (n = 50) for GM-CSF and 12% (n = 30) for TNF-α.

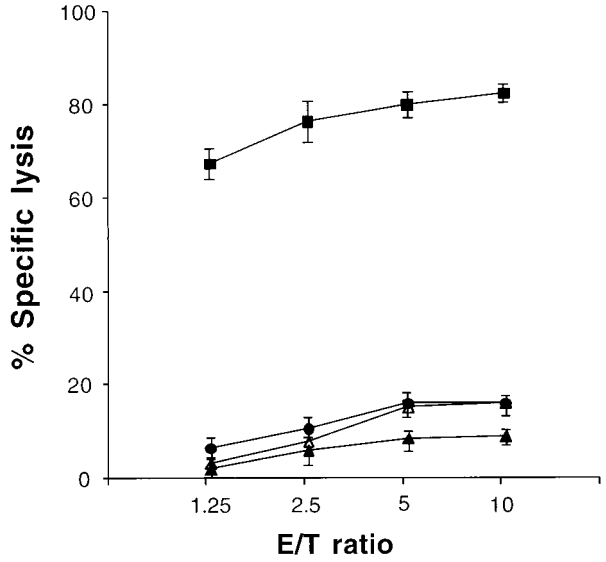

Fig. 2 Lysis of T2 cells pulsed with $p53_{264-272}$ peptide (LLGRNSFEV) by responder T cells derived from the PBLs of a healthy donor. T2 cells (△) were labeled with ^{51}Cr and preincubated with 10 μg/ml wild-type sequence $p53_{264-272}$ (■) or $p53_{149-157}$ (●) peptide for 1 h. They were then added to responder cells at the indicated ratios. K562 cells (▲) were used as targets to assess natural killer cell activity. The results shown are representative of three experiments.

Statistical Analysis. Student's *t* tests were used to interpret differences in CTL reactivities against different target cells and in the presence of blocking mAb.

Results

Induction of HLA-A2.1-restricted CTLs recognizing Wild-Type Sequence p53 Epitopes. To achieve effective induction of CTLs specific for the p53 peptides, we initially used DCs generated from PBMCs obtained from healthy HLA-$A2^+$ donors. Autologous CD8-enriched lymphocytes were repeatedly stimulated *in vitro* with DCs pulsed with the peptide. The DCs were generated in the presence of GM-CSF and IL-4 and were $CD1a^-$, $CD40^+$, $CD13^+$, $CD45^+$, $CD14^-$, $CD80^+$, $CD86^+$, and DR^+ (data not shown). The DCs were pulsed with either $p53_{149-157}$ or $p53_{264-272}$ peptide, irradiated, and used as stimulator cells. The CTL cultures were initiated in the presence of IL-6 and IL-12 (33, 34), and the responding cells were restimulated on day 7 with peptide-pulsed autologous DCs in a medium supplemented with IL-1α, IL-2, IL-4, Il-6, and IL-7 (Ref. 35; Fig. 1). Thereafter, the responding cells continued to be restimulated on a weekly basis with peptide-pulsed, adherent autologous PBMCs in the cytokine-supplemented medium. After two rounds of stimulation, the responding cells were tested for cytotoxic reactivity against peptide-pulsed target cells, but, usually, little to no specific reactivity was detected. After the fourth round of stimulation, however, the bulk effector cell population stimulated in the presence of the $p53_{264-272}$ peptide effectively lysed HLA-$A2^+$ T2 target cells pulsed with the relevant peptide (Fig. 2). Irrelevant peptide-pulsed and untreated target cells as well as K562 cells were not lysed by these effector cells. The CTL reactivity against peptide-pulsed-HLA-$A2^+$ T2 target cells was also evaluated using GM-CSF and TNF-α release assays. This bulk CTL cell line produced 225 pg/ml/24 h supernatant of GM-CSF in response to HLA-$A2^+$ T2 cells pulsed with the relevant peptide, $p53_{264-272}$, but produced <5 pg/ml in response to T2 cells alone or T2 cells pulsed with $p53_{149-157}$ peptide. TNF-α release was not detected. Using this culture system, bulk populations of anti-$p53_{264-272}$ CTLs were generated from PBMCs obtained from two of five healthy HLA-$A2^+$ donors. In contrast, none of the five bulk cultures of CD8-enriched T cells restimulated at least four times with $p53_{149-157}$ yielded effectors capable of recognizing peptide-pulsed targets.

Recognition of HLA-$A2^+$ Tumor Cells by Anti-p53 CTLs. After at least four rounds of restimulation, the ability of the bulk populations of effector cells to lyse HLA-$A2^+$ human tumor cells was evaluated. Among the tumor cell lines tested were the breast carcinoma cell line MCF-7, which was shown by Theobald *et al.* (20) to present the $p53_{149-157}$ and $p53_{264-272}$ epitopes, and SCCHN cell lines PCI-13, which has a p53 mutation in *p53* in codon 286 and can potentially present both epitopes, and OSC-19, which should not present $p53_{264-272}$ due to a mutation in codon 164 that generates a termination codon. As shown in Fig. 3, none of the human tumor cell lines tested was lysed by the bulk anti-$p53_{264-272}$ CTL line. Pretreatment of the target cells with IFN-γ alone or in combination with TNF-α to enhance MHC class I and antigen presentation (20) failed to sensitize any of these target cells to cytolysis by these anti-p53 CTLs. A similar lack of reactivity was observed in the GM-CSF-release assay (data not shown). Not unexpectedly, these antipeptide CTLs were not reactive against mitogen-activated lymphoblasts, which express a low but detectable level of p53. In parallel assays, the same tumor cells pulsed with the relevant peptide were lysed by these effector cells, with the exception of the SCCHN cell line PCI-4B, which is HLA-$A2^-$. This experiment indicated that the bulk population of anti-$p53_{264-272}$ CTLs could mediate cytolysis of these targets when they were presenting an artificially high level of the epitope.

Interestingly, one of the five bulk populations of effectors induced in the presence $p53_{149-157}$, none of which were reactive against peptide-pulsed targets, was cytotoxic against the SCCHN cell line PCI-13 (Fig. 4). This reactivity was blocked by anti-class I HLA mAb (data not shown). In addition, these effectors were not reactive against the HLA-$A2^-$ SCCHN cell line PCI-4B as well as K562, indicating that they were HLA-A2 restricted.

Isolation and Characterization of Anti-$p53_{264-272}$ CTL Clones. To facilitate further analysis of the anti-$p53_{264-272}$ CTLs, clones derived from the bulk populations of effectors were obtained by limiting dilution. After an analysis of the reactivity of more than 50 of these CTL clones against peptide-pulsed T2 cells, the more potent clones were expanded in culture. Of these, the clone designated p264#2 was the most stable and was selected for further characterization. A comparative analysis of the CTL clone and the bulk population showed that both mediated half-maximal lysis of peptide-pulsed target cells at a ligand concentration of approximately 10^{-9} M (Fig. 5), with the bulk population showing a slightly higher affinity than the CTL clone. The CTL clone was tested for reactivity against the p53-null HLA-$A2^+$ osteosarcoma

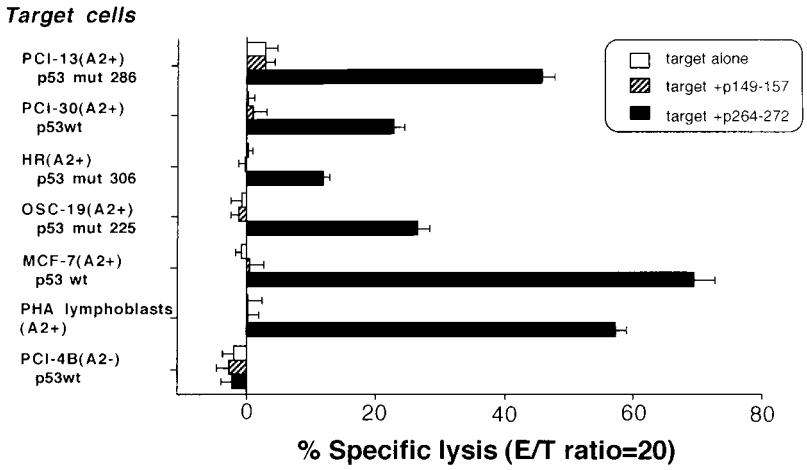

Fig. 3 Cytolytic activity of a bulk population of anti-$p53_{264-272}$ peptide-specific CTLs. Tumor targets (HLA-$A2^+$ and $A2^-$) and phytohemagglutinin-activated autologous lymphocytes were labeled with ^{51}Cr and tested for recognition by the CTL cell line alone or in the presence of the relevant $p53_{264-272}$ or irrelevant $p53_{149-157}$ peptide, as indicated. The status of p53 expressed by the target cells is also indicated. The results shown are representative of three experiments.

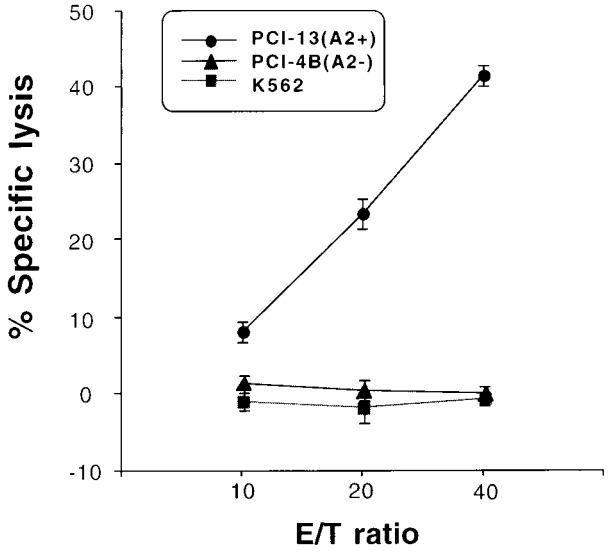

Fig. 4 Cytolytic reactivity of a bulk population of CTLs generated in the presence of DCs pulsed with the $p53_{149-157}$ peptide. Tumor targets (HLA-$A2^+$ and $A2^-$) were labeled with ^{51}Cr and tested for recognition by the CTL cell line. CTLs were added at an E:T ratio of 20:1. The results shown are representative of three experiments.

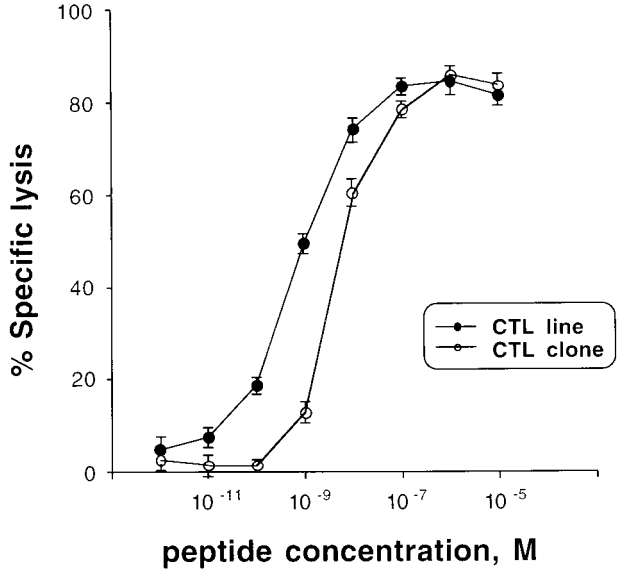

Fig. 5 Comparative analysis of reactivities of the bulk population and cloned anti-$p53_{264-272}$ CTLs against T2 target cells pulsed with various concentrations of the $p53_{264-272}$ peptide. CTLs were added at an E:T ratio of 20:1. The results shown are representative of two experiments.

SaOS-2 cell line, which is incapable of presenting p53-derived epitopes, and against SaOS-2cl3, a cloned cell line generated by the transfection of SaOS-2 cells with a mutated *p53*, which accumulates p53 protein. As indicated in Fig. 6, the CTL clone was not reactive against the p53-null parental cell line but was reactive against the p53 transfectant. This reactivity was blocked by anti-HLA-A2 mAb. A number of HLA-$A2^+$ human tumor targets were also tested for recognition by the CTL clone (Fig. 7). These included the PCI-13 and Daudi cell lines as well as the SCCHN cell lines SCC-4 and SCC-9. SCC-4 was shown by Ropke *et al.* (19) to be resistant to cytolysis by cloned human anti-$p53_{264-272}$ CTLs isolated in their laboratory, whereas SCC-9 was sensitive to cytolysis by cloned human anti-$p53_{264-272}$ CTLs. The CTL clone was cytolytic against SCC-9 and PCI-13 cells. As indicated in Fig. 7, reactivity against both targets was blocked by anti-class I HLA mAb. The SCC-4 and Daudi cells were not lysed by the cloned CTLs, further confirming the requirement of these CTLs for a p53-derived epitope and presentation by MHC class I molecules. Of note, whereas this CTL clone was reactive against human tumors accumulating p53 protein, it was not reactive against autologous mitogen-activated lymphocytes, which express higher levels of p53 than those detected in normal cells, but not as high as those found in tumor cells accumulating p53.

Isolation and Characterization of Anti-$p53_{149-157}$ CTL Clones. Two CTL clones reactive against $p53_{149-157}$-pulsed target cells as well as the HLA-$A2^+$ tumor cell line PCI-13 were isolated from the bulk population of effectors generated in the presence of the $p53_{149-157}$ epitope, which recognized PCI-13

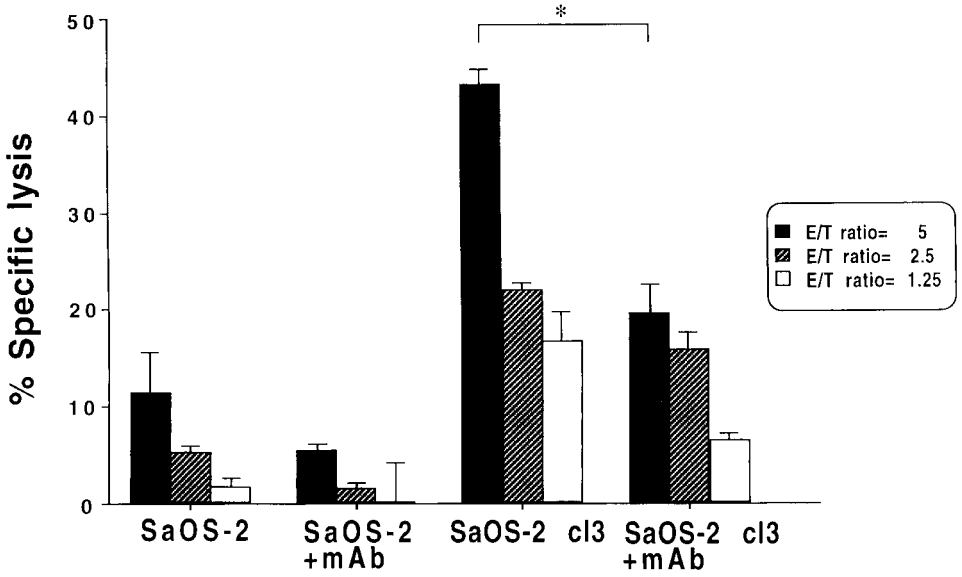

Fig. 6 Cytolytic reactivity of cloned anti-$p53_{264-272}$ against p53-null SaOS-2 cells and the cloned p53-transfected SaOS-2 cell line SaOS-2cl3. Targets were labeled with ^{51}Cr, and CTLs were added at the indicated E:T ratios in the presence or absence of anti-HLA-A2 mAb (MA2.1). *Asterisk* (*), significant ($P < 0.05$) mAb blocking of cytolytic reactivity against the transfectant cell line.

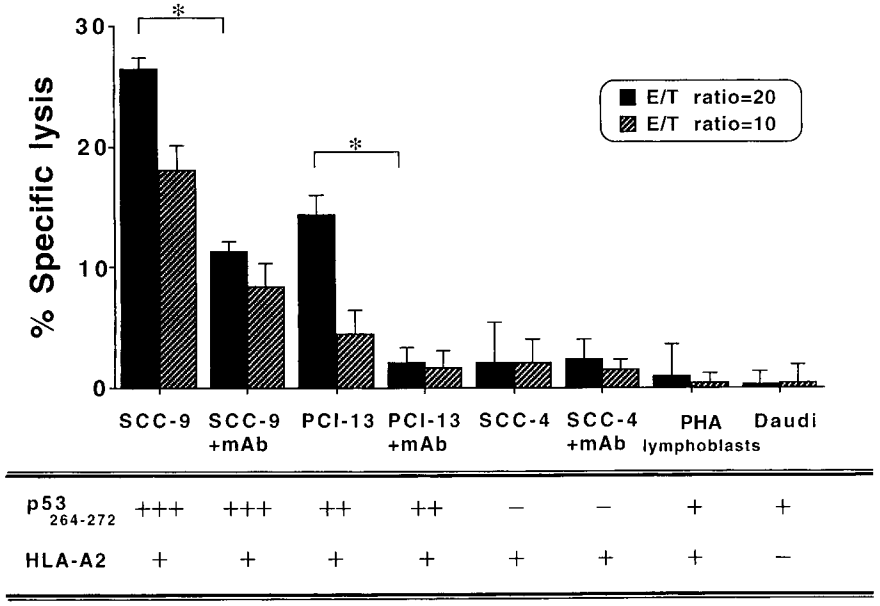

Fig. 7 Cytolytic reactivity of cloned anti-$p53_{264-272}$ CTLs against a panel of human normal and tumor cells. Targets were labeled with ^{51}Cr, and CTLs were added at the indicated E:T ratios in the presence or absence of anti-HLA-class I mAb (W6/32). *Asterisk* (*), significant ($P < 0.05$) mAb blocking of cytolytic reactivity against target cell lines.

but not the peptide-pulsed targets. Several other CTL clones were isolated that were reactive only against PCI-13, like the parental bulk population. None of the CTL clones or the parental bulk population of effectors was stable in culture, and further characterization of these populations was not possible.

Discussion

The objective of this study was the induction and characterization of human CTLs recognizing wild-type sequence p53 epitopes. Whereas we succeeded in achieving this objective, three aspects of our work are of particular importance because they identify potential obstacles to the successful development of p53-based immunotherapy: (*a*) CTLs recognizing the wild-type sequence $p53_{149-157}$ and $p53_{264-272}$ epitopes were induced from PBLs obtained from a minority of the normal donors tested; (*b*) only cloned populations of anti-p53 peptide-specific CTLs were capable of mediating cytolysis of tumor cells presenting the naturally processed epitope; and (*c*) accumulation of mutated p53 molecules by the tumor cell did not necessarily sensitize it to cytolysis by CTLs recognizing a wild-type sequence epitope.

The first point probably reflects the consequences of the "self" nature of the epitopes being targeted. The failure to induce and detect CTLs recognizing one or both wild-type sequence p53 epitopes from PBLs obtained from three of five normal donors might be due to a variety of reasons, ranging

from technical limitations of the methods being used to induce and detect these CTLs to biological events such as clonal deletion or anergy of T cells specific for these epitopes in the tested individuals. Ropke *et al.* (36) have reported that clonal ignorance or deletion relative to the $p53_{264-272}$ epitope was not apparent in PBLs obtained from normal donors, but the precursor frequency of these anti-p53 CTLs was low in the donors tested. It varied between 1:33,000 and 1:300,000. In this situation, short-term *in vitro* induction and detection of these CTLs are presumably limited.

The second point, which concerns the lack of reactivity against tumor cells of bulk anti-p53 peptide-specific CTL populations, might reflect differences in the avidities of bulk and cloned CTLs for the ligand. However, in the case of the anti-$p53_{264-272}$ CTL populations evaluated in this study, bulk and cloned CTLs mediated half-maximal lysis of peptide-pulsed target cells at a similar peptide concentration (10^{-9} M). This is comparable to the affinities reported for CTLs recognizing other tumor-associated wild-type sequence peptides, such as those derived from melanoma antigen recognized by T cells, melanoma antigen-encoding gene, and gp100 (37). Therefore, despite the use of DCs for *in vitro* induction, the potency of the CTLs recognizing the wild-type sequence p53 epitopes does not appear to be optimal. The data obtained from $p53^{-/-}$ A2.1/K^b transgenic mice (21) suggest that a low frequency of clones capable of recognizing only very low copy numbers of p53-derived epitopes is due to tolerance circuits. It is presently unclear whether the T-cell repertoire in healthy individuals that can recognize these epitopes is similarly restricted. Hence, the use of DCs with an ever-decreasing density of peptide in sequential rounds of restimulation might allow growth of the rare, high-avidity anti-p53 CTLs. Alternatively, DCs genetically modified to express p53-derived epitopes rather than peptide-pulsed DCs might induce anti-p53 CTLs with potentially more potent antitumor reactivity. A vaccine consisting of genetically modified DCs expressing the H-$2K^d$-restricted wild-type sequence murine $p53_{232-240}$ has already been shown to induce antitumor CTLs as well as tumor rejection in mice (38), whereas human DCs transfected with a variety of genes encoding CTL-defined tumor antigens were found to be effective in the *in vitro* induction of antitumor CTLs (39).

The last point relates to the lack of sensitivity of certain tumor cells and p53-transfected cells to cytolysis mediated by anti-$p53_{264-272}$ CTL clones (19, 40). Whereas a general defect in antigen processing and presentation occurring in these cells could explain this observation (41), other possibilities exist. Initially, the accumulation of mutant p53 molecules in tumor cells was considered to be a prerequisite for the presentation of p53 epitopes and recognition by the CTLs. However, it has become apparent in this study and in other reports (19, 40) that not all cells accumulating mutant p53 molecules and presumably capable of processing and presenting wild-type sequence p53 epitopes are sensitive to CTLs recognizing these epitopes. It has been recently demonstrated that the missense mutation at p53 codon 273 blocks processing of the $p53_{264-272}$ epitope from p53 molecules expressing this mutation. Consequently, cells accumulating p53 molecules expressing this mutation are not recognized by anti-$p53_{264-272}$ CTLs (40). As additional CTL cell lines recognizing the presently known CTL-defined wild-type sequence p53 epitopes as well as class II-restricted epitopes and other class I-restricted epitopes are generated and characterized and the factors affecting p53 stability and antigen presentation in various tumors are further defined, a more definitive pattern relating p53 alterations/mutations to the presentation and T-cell recognition of defined p53 epitopes should become evident. This relatively novel aspect of antigen presentation might be a crucial component in immunoselection of p53 antigen epitope loss variants (8) and the applicability of p53-based immunotherapy.

Whereas wild-type sequence p53 epitopes are leading candidates in the development of broadly applicable cancer vaccines, other transformation-related antigens, which are overexpressed in tumors, have been identified for potential vaccine development (42). The development of "general tumor vaccines" (20), including p53-based vaccines, that would target a variety of transformation-related gene products overexpressed in tumors of diverse histologies is currently one of the major goals of cancer immunotherapy.

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