The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product

(murine tumor antigens/cytotoxic T lymphocytes/endogenous murine leukemia virus/tandem mass spectrometry/major histocompatibility complex class I peptides)

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Communicated by Lloyd Old, Ludwig Institute for Cancer Research, New York, NY, May 2, 1996 (received for review February 15, 1996)

ABSTRACT Tumors express peptide antigens capable of being recognized by tumor-specific cytotoxic T lymphocytes (CTL). Immunization of mice with a carcinogen-induced colorectal tumor, CT26, engineered to secrete granulocyte/macrophage colony-stimulating factor, routinely generated both short-term and long-term CTL lines that not only lysed the parental tumor *in vitro*, but also cured mice of established tumor following adoptive transfer *in vivo*. When either short-term or long-term CTL lines were used to screen peptides isolated from CT26, one reverse-phase high performance liquid chromatography peptide fraction consistently sensitized a surrogate target for specific lysis. The bioactivity remained localized within one fraction following multiple purification procedures, indicating that virtually all of the CT26-specific CTL recognized a single peptide. This result contrasts with other tumor systems, where multiple bioactive peptide fractions have been detected. The bioactive peptide was identified as a nonmutated nonamer derived from the envelope protein (gp70) of an endogenous ecotropic murine leukemia provirus. Adoptive transfer with CTL lines specific for this antigen demonstrated that this epitope represents a potent tumor rejection antigen. The selective expression of this antigen in multiple non-viral-induced tumors provides evidence for a unique class of shared immunodominant tumor associated antigens as targets for antitumor immunity.

CD8⁺ cytotoxic T lymphocytes (CTL) recognize antigenic peptides expressed in the groove of cell surface major histocompatibility complex (MHC) class I molecules. Extensive studies evaluating viral and minor histocompatibility antigens have demonstrated that these antigenic peptides are 8–10 amino acids long and are derived from the degradation of intracellular proteins (1–3). Because of various genetic alterations leading to transformation, tumor cells may express the products of mutated, amplified, or reactivated genes, which can be seen by the immune system as potential rejection antigens. Examples of these antigens have now been identified in both murine and human tumors. Using CTL clones and DNA cloning techniques, several melanoma related tumor antigens as well as a mouse mastocytoma tumor antigen have been described (4–13). Direct peptide isolation and sequencing have lead to the identification of a murine lung carcinoma antigen and a human melanoma antigen (8, 9).

For any tumor antigen defined by T cells, it is critical to ascertain its relevance to *in vivo* antitumor responses. Oper-

ationally, this is best accomplished by demonstrating that either (i) T cells specific for the antigen will reject tumors *in vivo* after adoptive transfer, or (ii) vaccination with the antigen (as peptide, protein, or recombinant nucleic acid) will generate systemic immunity capable of protecting against growth of either challenge or preestablished tumor. The ultimate utility of any antigen in formulating a cancer vaccine not only depends on how efficiently it is presented by a tumor's MHC, but also on the preexisting T-cell repertoire specific for that antigen. In addition to the antigen itself, the efficacy of a tumor vaccine will also depend upon how that antigen is presented to the immune system during initiation or priming of the response.

We have previously proposed that tumor antigens identified by T cells induced upon immunization with whole cell vaccines would define a set of immunodominant targets. We have tested this postulate by using a carcinogen-induced murine colon tumor, CT26.

Here we report that short-term as well as long-term cultures of tumor-specific CTL can be readily obtained from mice immunized with CT26 genetically engineered to secrete granulocyte/macrophage colony-stimulating factor (GM-CSF). These CTL lines identified a single immunodominant peptide species derived from the envelope protein (gp70) of an endogenous ecotropic murine leukemia virus (MuLV), *env-1*. Adoptive transfer experiments indeed confirm that this antigen is a biologically relevant target for antitumor immunity. The presence of such an immunodominant tumor antigen suggests that antigen-specific vaccine strategies may be designed to target a small number of immunodominant antigens. Furthermore, these findings provide a basis for the exploration of endogenous retrovirus encoded products as potential antigenic targets in human malignancies.

MATERIALS AND METHODS

Tumor Cells. CT26 is a colon epithelial tumor derived by intrarectal injections of *N*-nitroso-*N*-methylurethane in BALB/c mice (14, 15). CT26/GM-CSF was derived as described (16). CT26/B7 was derived by cotransfection of CT26 with plasmids encoding the human B7-1 and a hygromycin-selectable marker. P815 is a mastocytoma cell line derived

Abbreviations: RP-HPLC, reverse-phase high performance liquid chromatography; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; GM-CSF, granulocyte/macrophage colony stimulating factor; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; MuLV, murine leukemia virus; RT-PCR, reverse transcription-PCR.

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from DBA/2 mice. MC57GL^d was obtained by transfecting the fibrosarcoma line MC57G with pL^d.444, a plasmid containing the gene encoding the L^d molecule and a neomycin-selectable marker. B16F10 (H-2^b) is a murine melanoma cell line, and B78H1 is a variant of B16 that has lost MHC class I expression (17). All tumor cell lines were cultured as described (16).

Generation of CTL Lines. BALB/c mice were immunized s.c. with 10⁶ irradiated (50 Gy) CT26/GM-CSF cells. Two weeks later, splenocytes from immunized mice were isolated and stimulated *in vitro* with mitomycin (50 µg/ml)-treated CT26 in the presence of 10 units of interleukin 2 per ml (16). For short-term cultures, T cells were stimulated for one or two *in vitro* stimulations. For CTL lines, viable T cells were restimulated with fresh, mitomycin-treated CT26/B7 cells every 7–14 days.

Cell Preparation. For peptide isolation, CT26 cells were grown s.c. in BALB/c mice. Methods of isolating primary colonic and small intestinal epithelial cells have been described (18).

Peptide Isolation and Purification. CT26 tumor explants were lysed by one of two methods. In the first method, whole cell lysates were obtained and purified as described (19) using 0.1% trifluoroacetic acid (TFA), and separated on a C₁₈ silica reverse-phase high-performance liquid chromatography (RP-HPLC) column (Vydac) using 0.1% TFA/H₂O (buffer 1) and 0.1% TFA/acetonitrile (buffer 2) at 2% buffer 2/min gradient. In the second method, the surface MHC class I molecules were isolated by affinity column purification (8). The cell lysate was sequentially loaded onto three protein A Sepharose affinity columns binding either an irrelevant antibody, OKT8 (anti-human CD8), anti-K^d and I^d monoclonal antibody 3412S, or anti-L^d antibody 3057S. Peptides were eluted with 0.2 M acetic acid and fractionated using a reverse-phase C₁₈ column (G18-032 Brownlee; Rainin Instruments) with 0.1% heptafluorobutyric acid (HFBA) in water and 0.085% HFBA in 60% acetonitrile in water (buffer B). The gradient consisted of 100% buffer A (0–6 min), 0 to 15% buffer B (6–11 min), 15 to 60% buffer B (11–61 min), and 60–100% buffer B (61–68 min) at a flow rate of 200 µl/min. The bioactive fraction was rechromatographed using TFA instead of HFBA as the organic modifier.

Peptide Screening. ⁵¹Chromium-labeled P815 and MC57GL^d cells were pulsed with 5% of peptide fractions and evaluated in a 4-h ⁵¹chromium release assay (19).

Determination of Candidate Peptides by Mass Spectrometry. Second-dimension bioactive fractions from affinity column purifications were separated with an on-line microcapillary effluent splitter (8).

Amino Acid Sequence Analysis by Mass Spectrometry. Collision-activated dissociation mass spectra of ions from candidate peptides were recorded on a TSQ 7000 triple-quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) as described (8, 20).

Peptide Synthesis. The following peptides were synthesized using standard Fmoc (*N*-α-9-fluorenylmethoxycarbonyl) chemistry with a Gilson AMS422 peptide synthesizer: NP147-155 (TYQRTRALV) (1), NP366-374 (ASNENMETM) (1), AH1 (SPSYVYHQF), and QL9 (QLSPFPFDL) (21).

PCR Primers and Reverse transcription-PCR (RT-PCR). The 5' and 3' primers used were 5'-ACCTTGTCGGAAGT-GACCG-3' and 5'-GTACCAATCCTGTGTGGTTCG-3', respectively. For RT-PCR, total RNA was obtained using the TRIzol Reagent (GIBCO/BRL). One microgram of the RNA was reverse transcribed and the cDNAs were amplified with *Taq* polymerase at an annealing temperature of 62°C and denaturing temperature of 94°C for 40 cycles.

RESULTS

CTL Lines Recognized a Single Peptide Fraction Derived from CT26 Cells. Short-term (one or two *in vitro* stimulations)

FIG. 1. Short-term and long-term CTL lines from CT26/GM-CSF immunized mice are specific for the parental CT26 tumor. Short-term CTL cultures were tested against CT26 target after one (○) and two (□) *in vitro* stimulations. A long-term CTL line was tested for lysis against CT26 (●), CT26B7 (■), P815 (△), and MC57G (▲). % Specific lysis = 100% × (sample cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm). % CD8-blockable lysis = (% lysis without anti-CD8 antibody) – (% lysis with anti-CD8 antibody).

CT26-specific CTL can be routinely generated from splenocytes isolated from CT26/GM-CSF immunized mice. These T cells demonstrated tumor-specific lytic activity against CT26 that are both CD8 (Fig. 1) and MHC class I-blockable (data not shown). The short-term anti-CT26 CTL can be continuously passaged *in vitro* using CT26/B7 as stimulator cells (22). The resultant long-term anti-CT26 CTL lines (>5 passages) recognized CT26 and CT26/B7 equivalently, demonstrating that the presence of B7-1 in the culture did not alter the specificity of the CTL (Fig. 1). In addition, long-term CTL lines failed to recognize the allogeneic fibrosarcoma line, MC57G, and minimally recognized the MHC-matched DBA/2-derived mastocytoma line, P815 (Fig. 1). Furthermore, the long-term CTL lines expressed predominantly CD8 (>98%) and CD3 (>98%), as determined by flow cytometry (data not shown).

CT26 is MHC class I⁺ after *in vitro* passage, and the expression level is retained in the primary tumor explant after *in vivo* passage (23). To examine the diversity of peptide species recognized by the overall population of CT26-specific CD8⁺ cells, we began by using short-term CTL cultures from immunized mice to survey RP-HPLC peptide fractions from CT26 lysates. In contrast to long-term lines and clones, in which significant repertoire skewing can occur during repetitive stimulations, short-term cultures should most closely reflect the spectrum of *in vivo* CD8⁺ activity. When peptides extracted from 10¹⁰ freshly explanted CT26 tumor cells by whole cell acid extraction with TFA were fractionated on RP-HPLC, one peptide fraction (Fig. 2) consistently sensitized P815 cells for lysis by the primary anti-CT26 CTL. Titration experiments demonstrated that the bioactive fraction retains its ability to sensitize the surrogate target after more than a 1000-fold dilution (data not shown). This single bioactive

FIG. 2. Identification of one immunodominant RP-HPLC fraction. One RP-HPLC fraction obtained from CT26 whole cell TFA extraction was detected as bioactive in the ⁵¹Cr-release assay using a short-term CTL population. The dashed line denotes the hydrophobic gradient.

fraction has been observed reproducibly in at least 15 separate CT26 cell preparations and short-term CTL cultures. The dominant bioactivity eluted in a single RP-HPLC fraction throughout multistep purification using different elution conditions (Fig. 3 *a* and *b*). When long-term lines were used to survey peptide fractions, the same bioactive peak was observed with no additional subdominant reactivities emerging. Taken together, these results suggest that the majority of the CD8⁺ response to CT26 is specific for a single peptide.

To determine the MHC locus responsible for presenting the bioactive peptide species to the CTL, serial affinity column purifications were performed using a series of locus-specific, anti-MHC class I antibodies. Peptides eluted from the L^d molecules contained a bioactive species with the same HPLC elution profile as that observed with whole cell acid extraction. In contrast, peptides isolated from the anti-K^d/D^d column or an isotype-matched irrelevant antibody affinity column failed to sensitize P815 targets for lysis by CT26-specific CTL lines (data not shown). Both short-term and long-term CTL lines lysed P815 targets pulsed with the same L^d-associated peptide fraction.

Identification and Sequencing of the Bioactive Peptide. To identify the sequence of the immunodominant peptide by mass

spectrometry, affinity purification was performed to obtain less complex RP-HPLC fractions. L^d affinity column purified peptides were fractionated by RP-HPLC using HFBA as the organic modifier. Individual fractions were analyzed for sensitization of P815 targets by ⁵¹Cr release assay using long-term CTL lines. As was seen in the whole cell extraction experiment with the short-term CTL (Fig. 2), a single bioactive fraction (Fig. 3*a*, peak 1) was observed. This fraction was rechromatographed using a different organic modifier (TFA). This second-dimension fractionation again produced a single bioactive peak spanning two fractions (Fig. 3*b*, peaks 2 and 3). As assessed by electrospray ionization tandem mass spectrometry, each fraction still contained more than 100 distinct peptide species. To determine which of these peptides was the antigenic CT26 peptide, peaks 2 and 3 were separately rechromatographed by microcapillary HPLC. Using an on-line microcapillary effluent splitter system (8), five-sixths of the effluent was directed into the mass spectrometer, while one-sixth was simultaneously collected into a 96-well microtiter plate for subsequent ⁵¹Cr release assay. This allowed us to correlate bioactivity with ion abundance for individual peptides observed in the mass spectrum. The splits of both peak

FIG. 3. Determination of the antigenic peptide sequence by mass spectrometry. (*a*) Peptides were isolated from an L^d affinity column and fractionated using RP-HPLC with HFBA as the organic modifier (●). Bioactive peak 1 was identified using a long-term CTL line. As a control, peptides recovered from a control affinity column were also fractionated and tested (▲). (*b*) Peak 1 was rechromatographed using TFA as the organic modifier. The bioactivity was found in peaks 2–3 (●). Nonbioactive fractions 21–26 from *a* were combined, rechromatographed, and screened (▲). (*c* and *d*) Candidate peptides were chosen by comparing ion abundance to CTL activity, following a postcolumn effluent split of the second RP-HPLC purified peaks 2 and 3. Summation of mass spectra is shown for peptides deposited in active wells for peak 2 (*c*) and peak 3 (*d*). Full scale is 10^6 (*c*) and 10^7 (*d*) counts. Results of the ⁵¹Cr-release assays are shown (*) in Insets of *c* (peak 2) and *d* (peak 3). Peptide ion abundance is shown for the peptide with *m/z* value of 564 (▲) for peaks 2 and 3 in Insets of *c* and *d*, respectively. Full scale is 7.5×10^5 (Inset of *c*) and 1.3×10^6 (Inset of *d*) counts. (*e*) Collision-activated dissociation mass spectra of $(M + H)^{2+}$ ions with *m/z* 564. Predicted masses for fragment ions of types *b* and *y* (24) are shown above and below the deduced amino acid sequence. Ions observed in the spectrum are underlined. Ions that represent single and multiple losses of water are denoted with an ●.

2 and peak 3 produced CTL activity spanning two consecutive wells. Although many peptides were present in these bioactive fractions (Fig. 3 *c* and *d*), the relative ion abundance of only one of these peptides [with mass to charge ratio (m/z) of 564] matched the activity profile of the ^{51}Cr -release assays (Fig. 3 *c* and *d*, *Inset*). Approximately 10 fmol of m/z 564 was used to obtain collision-activated dissociation spectra of this peptide. Analysis of the mass spectrum (Fig. 3*e*) revealed the sequence, SPSYVYHQF (referred to from hereafter as AH1). The total mass of AH1 is 1128, consistent with the notion that the 564 signal represents the doubly charged species. A search of the National Center for Biotechnology Information and GenBank data bases identified one protein that contains a peptide with a 100% homology to AH1. This protein was gp70, the *env* gene product of the endogenous ecotropic MuLV, *env-1*, a member of the mammalian C-type retroviruses. The gp70 epitope corresponding to AH1 is gp70(423–431).

The identity of AH1 as the bioactive peptide recognized by CT26-specific CTL was confirmed by demonstrating that synthetic peptide corresponding to the deduced sequence sensitized the surrogate target for lysis by an anti-CT26 CTL line (Fig. 4). The CTL line recognized AH1 at a concentration as low as 5 pM. None of the control L^d, K^d, or D^b binding peptides sensitized MC57G/L^d for lysis by the CT26-specific CTL over a wide range of peptide concentrations. In addition, the synthetic AH1 peptide coelutes with the CT26 bioactive fraction on RP-HPLC analysis (data not shown).

We next screened the expression pattern of the MuLV *env* transcript in a variety of normal tissues and cell lines. A set of primers were designed to span the epitope with an expected size of 594 bp. The sequences of the primer sets were determined based on the published homologous sequences among the MuLV isolates in the AKR (25), C57BL/6 (26), and BALB/c mice (27). RT-PCR of RNA isolated from either *in vitro* cultured CT26, CT26B7, or *in vivo* explanted CT26 expressed the *env* gene transcript (Fig. 5). B16 as well as its MHC class I⁺ variant, B78H1, also express the MuLV *env* transcript (26). MC57G and MC57GL^d, on the other hand, did not express detectable levels of the *env* transcript. Interestingly, RT-PCR of P815 demonstrated a weakly positive band, suggesting that it expresses a low level of the *env* transcript. This may explain the occasional elevated background in peptide screening assays when P815 was used as a surrogate target.

Peptides from BALB/c colonic and small intestinal epithelia were obtained to test whether AH1 is normally expressed on colonic epithelium. Bioactive peptides cannot be detected in RP-HPLC fractions containing peptides from either of two normal tissues (data not shown). In contrast, peptides isolated

FIG. 4. Functional confirmation of the AH1 sequence. An anti-CT26 CTL line was tested for lysis against MC57GL^d targets sensitized with various concentrations of AH1 (○). As controls, the same targets were pulsed with either K^d-restricting influenza nucleoprotein peptide, NP147–155 (□), D^b-restricting influenza nucleoprotein peptide, NP366–374 (■), or L^d-restricting peptide, QL9 (△). The 4-h ^{51}Cr -release assay was carried out at an effector-to-target cell (E/T) ratio of 5:1.

FIG. 5. RT-PCR of MuLV *env* transcript in tumor lines. RT-PCR was performed on total RNA isolated from cultured cells and *in vivo* grown CT26 tumor. RT-PCR of the β_2 -microglobulin ($\beta_2\text{M}$) transcript was used as an internal control.

from an equivalent number of CT26 cells contained the AH1 peptide recognized by the CTL line (data not shown). RT-PCR of tissue samples isolated from 6- to 8-week-old as well as >8-month-old mice also failed to detect any *env* gene transcript (data not shown). In addition to epithelial cells isolated from gastrointestinal organs, testes, thymic stroma, and bone marrow-derived thymic elements were also completely negative for the *env* gene transcript.

In Vivo Demonstration of AH1 as a Tumor Rejection Antigen. To test whether the AH1-specific CTL lines are effective against the parental tumor *in vivo*, an adoptive transfer experiment was performed into mice bearing established CT26 tumor subcutaneously. Three days after receiving live CT26, mice were injected (i.v.) with AH1-specific CTL. Eighty percent of the mice remained tumor-free after 6 weeks, as compared with only 40% of mice in the group that received the CT26/GM-CSF vaccine (Fig. 6). In contrast, all mice receiving either saline, irradiated CT26, or primary splenocytes from mice receiving irradiated CT26/GM-CSF vaccine cells developed tumors within 3 weeks. The finding that the AH1-specific CTL lines are effective in eradicating established CT26 *in vivo* strongly suggests that AH1 represents a relevant target antigen for *in vivo* antitumor immunity. AH1-specific T-cell lines are currently being cloned for the purpose of confirming the immunodominance of this antigen. Interestingly, exogenous interleukin 2 was not required for CTL activity *in vivo*, unlike what has been previously reported (28, 29). Evaluation of cytokine release demonstrated that the CTL lines produce interleukin 2, tumor necrosis factor α , and GM-CSF in response to CT26 *in vitro* (data not shown). Using a panel of V β - and V α -specific monoclonal antibodies, a predominance in V β 8.3 was detected in at least five independently derived CTL lines (data not shown).

FIG. 6. Adoptive transfer of AH1-specific CTL line can cure mice with established CT26 tumors. Mice were injected with 2×10^4 live CT26 s.c. in the right flank. Mice were rescued 3 days later with 10^6 irradiated CT26/GM-CSF (○) s.c., 10^6 irradiated CT26 (□) s.c., saline (△) i.v., 1.5×10^7 CT26/GM-CSF-immunized fresh splenocytes (▲) i.v., or 7×10^6 AH1-specific CTL (●) i.v. Mice were monitored twice a week for the development of tumor.

Therefore, the majority of T cells in the long-term CTL lines specific for AH1 appear to use a limited set of V β s.

DISCUSSION

The deduced AH1 sequence, SPSYVYHQF, is derived from gp70, one of two *env* gene products of the endogenous MuLV, and corresponds to amino acids 423–431. AH1 conforms to the published motifs of known L^d-binding peptides, including self peptides (30), viral peptides (31), alloantigens (2), and tumor peptide antigens (10).

The *env* gene products of MuLV (32) are expressed in a variety of tumor cells, including B16 melanoma (26), lymphomas and leukemias of high MuLV titer strains of mice (33–35), and simian virus 40-transformed NIH 3T3 cells (36). We have found that (i) CT26 expresses the MuLV *env* gene product while it is silent in the normal tissues of BALB/c mice, and (ii) this viral antigen, gp70, serves as a potential tumor rejection antigen for the immune system. Our findings are consistent with that of DeLeo *et al.* (37) who previously demonstrated the surface expression of gp70 on many tumors of known viral etiology but not on normal mouse tissues. In addition, the gp70 gene product is not expressed in the thymus which explains why central tolerance is not observed to this endogenous gene product. Chen *et al.* (38) described a similar pattern of immunogenicity of a thymus leukemia antigen. In strains of mice in which this antigen is not expressed in the thymus, immune responses are generated. However, in mice that demonstrated thymic expression, tolerance is observed. It is not clear whether the expression of the MuLV *env* gene product was critically involved in the initiation of transformation or tumorigenesis, or was the result of genetic dysregulation associated with the transformation process. Whether or not *env* induction is involved in the transformation process does not necessarily determine its utility as a tumor rejection antigen.

DeLeo *et al.* (39) have suggested a relationship between tumor-specific transplantation antigens (TSTA) expressed by chemically induced sarcomas and the gp70 surface expression of Moloney-MuLV. Lennox and coworkers (40, 41) demonstrated *in vivo* protection against transplantable gp70-expressing tumors, further suggesting that the specificities of TSTA are carried on gp70-like molecules. However, this finding that gp70 is expressed in different tumors and encodes for TSTA presents a paradox in light of observations that tumors arising from the same chemical carcinogens often do not express shared dominant rejection antigens based on cross-protection assays (39–41). We are actively investigating the utility of gp70 proteins or AH1 peptide as *in vivo* rejection antigens in other tumor systems.

Retrovirally encoded proteins have previously been reported to be potential immune reactive antigens expressed by tumors. gp70, in particular, has been demonstrated to be a CD4⁺ T cell antigen expressed by a murine Friend leukemia virus–MuLV induced T-cell leukemia (42, 43). However, AH1 is the first reported tumor antigen that derives from the expression of the MuLV *env* gene product in a chemically induced colon tumor. The relevance of this finding for human tumor antigens remains to be examined. There have been many reports demonstrating the existence of MuLV-like sequences in the human genome (44–47). More than one study has demonstrated the expression of human endogenous retroviral gene products including the expression of the *gag* gene in the sera of patients with human seminoma (48) and renal cell adenocarcinoma (49), and the expression of the *pol* gene in human breast (50) and colorectal carcinomas (51). The induction of expression of endogenous retroviral gene products is thought to occur as a result of either transcriptional activation, or retrotransposition of the proviral DNA with subsequent promoter insertion and oncogene activation or suppresser gene inactivation. Several murine and human endogenous

retroviruses have been reported to be transcriptionally active. For example, transcriptional regulation of expression of the human endogenous retroviral gene product, HERV-K, has been demonstrated in the hormone-sensitive human breast tumor line, T47D (52, 53). Although endogenous retroviral gene products have not yet been shown to be T-cell antigenic targets expressed by human tumors, antibodies to a HERV-K outer membrane protein have been found in human sera (54). Furthermore, the expression of a chemotactic inhibitory protein, which has an immunological cross-reactivity to the MuLV envelope protein p15E, has been detected in the effusions of patients with squamous cell carcinoma, melanoma, and carcinoma of the bladder (55). Taken together, these findings support the further analysis of endogenous retroviral gene products as immunologically relevant human tumor antigens.

Independently generated short-term anti-CT26 CTL lines reproducibly recognized one single HPLC fraction in multiple peptide preparations. This result suggests that CT26/GM-CSF immunization has resulted in the generation of CTL *in vivo* which focus on a limited subset of a large array of potential antigenic peptides. As assessed by the RP-HPLC profile and mass spectrometry, the level of AH1 expression was not overly abundant compared with that of other L^d-associated peptides (Fig. 3 *c* and *d*). This is in contrast to the murine lung carcinoma system (9) where an antigenic peptide was found in sufficient abundance to allow sequence analysis by Edman degradation. Although it is unlikely that the dominant immune response to AH1 peptide can be explained by an unusually high overall affinity for its T-cell receptor, studies are underway to test this possibility. Other explanations for the preferential reactivity against AH1 may involve either a higher percentage of T cells within the T-cell repertoire with specificity toward AH1, or the presence of additional immunodominant CD4⁺ T-cell epitopes on gp70. Limiting dilution analysis of AH1-specific T cells will be needed to address the former possibility.

Our finding that AH1 represents the single immunodominant tumor-associated MHC class I-restricted peptide antigen of CT26 is also significant in light of observations made in other systems where multiple bioactive peptide fractions are detected (8, 56, 57). In these studies, the biological relevance of the antigens identified in each bioactive fraction as *in vivo* rejection antigens could not easily be determined. Demonstrating the existence of immunodominant rejection antigens is a critical first step toward designing recombinant antigen-specific vaccines that are generalizable to most patients with cancer.

In normal BALB/c mice, immunization with CT26 cells were not able to significantly enhance the activation of T cells above the threshold necessary for rejection of subsequent CT26 tumor challenge. In our system, however, priming with GM-CSF producing CT26 vaccine cells has overcome potential mechanisms of tolerance (anergy, ignorance, deletion, or suppression) to endogenous antigen expression. This is a critical requirement for any vaccine approach that is expected to be effective therapy for patients with cancer. If antigen-specific vaccines are to have therapeutic utility, they must also demonstrate *in vivo* priming against tumors that express endogenous antigens. Preclinical models predict that the induction of effective antitumor immune responses depends on at least three parameters including (i) how efficiently an antigen is presented by a tumor's MHC, (ii) the preexistence of a T-cell repertoire specific for the tumor rejection antigen, and (iii) how efficiently the antigen is presented to the immune system during the induction of the immune response. Several groups have demonstrated successful immune priming with model MHC class I antigens either pulsed on dendritic cells (58) or mixed with various adjuvants (59). However, preliminary vaccination studies with either AH1-pulsed bone marrow-derived dendritic cells or AH1 peptide in incomplete Freund's adjuvant demonstrated a minimal reduction in the growth of subsequent tumor challenge compared with a control peptide

(data not shown). Several possibilities may account for the failure of the AH1 peptide to elicit a potent immune response against CT26 *in vivo*: (i) The methods and conditions of peptide immunization were not optimal in eliciting a potent, protective immunity, and (ii) The MHC class I-restricted AH1 peptide alone may not be sufficient in eliciting a protective immune response. Rather, both the CD4⁺ T-cell epitope combined with the CD8⁺ T-cell epitope may be required. In support of this second explanation is data from preclinical models demonstrating that both CD4⁺ and CD8⁺ T cells are required as effectors for mediating potent antitumor immune responses (16, 60). To test this hypothesis, we are actively pursuing the identification of the immunodominant CD4 antigen expressed by CT26.

We thank A. Adler and G. Yochum for technical advise, J. Schneck for providing QL9 peptide, D. Margulies for providing pL^d.444, and M. Jenkins for providing pcEXV-3 B7-1. E.M.J. is the recipient of the National Institutes of Health K11 Physician-Scientist Award (CA01692) and a Specialized Program of Research Excellence in Gastrointestinal Malignancies grant (CA62924). P.H.G. is supported by U.S. Public Health Service Grant AI33993. A.S.W. is supported by Grant DIR 90-16567 of the National Science Foundation. W.W. is supported by U.S. Public Health Service Training Grant AI07496.

- Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G. & Rammensee, H. G. (1990) *Nature (London)* **348**, 252-254.
- Udaka, K., Tsomides, T. J. & Eisen, H. N. (1992) *Cell* **69**, 989-998.
- Van Bleek, G. M. & Nathenson, S. G. (1990) *Nature (London)* **348**, 213-216.
- Tsomides, T. J. & Eisen, H. N. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3487-3489.
- Van den Eynde, B., Lethe, B., Van Pel, A., De Plaen, E. & Boon, T. (1991) *J. Exp. Med.* **173**, 1373-1384.
- Coulie, P. G., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, C., Szikora, J. P., Renauld, J.-C. & Boon, T. (1994) *J. Exp. Med.* **180**, 35-42.
- Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., Miki, T. & Rosenberg, S. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3515-3519.
- Cox, A. L., Skipper, J., Chen, Y., Henderson, R. A., Darrow, T. L., Shabanowitz, J., Engelhard, V. H., Hunt, D. F. & Slingluff, C. L., Jr. (1994) *Science* **264**, 716-719.
- Mandelboim, O., Berke, G., Fridkin, M., Feldman, M., Eisenstein, M. & Eisenbach, L. (1994) *Nature (London)* **369**, 67-71.
- Lurquin, C., Van Pel, A., Mariame, B., De Plaen, E., Szikora, J. P., Janssens, C., Reddehase, M. J., Lejeune, J. & Boon, T. (1989) *Cell* **58**, 293-303.
- Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P. & Boon, T. (1993) *J. Exp. Med.* **178**, 489-495.
- Coulie, P. G., Lehmann, F., Lethe, B., Herman, J., Lurquin, C., Andrawiss, M. & Boon, T. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7976-7980.
- Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H. & Beach, D. (1995) *Science* **269**, 1281-1284.
- Griswold, D. P. & Corbett, T. H. (1975) *Cancer* **36**, 2441-2444.
- Corbett, T. H., Griswold, D. P., Jr., Roberts, B. J., Peckham, J. C. & Schabel, F. M., Jr. (1975) *Cancer Res.* **35**, 2434-2439.
- Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. & Mulligan, R. C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3539-3543.
- Levitsky, H. I., Lazenby, A., Hayashi, R. J. & Pardoll, D. M. (1994) *J. Exp. Med.* **179**, 1215-1224.
- Evans, G. S., Flint, N., Somers, A. S., Eyden, B. & Potten, C. S. (1992) *J. Cell Sci.* **101**, 219-231.
- Woods, A. S., Huang, A. Y. C., Cotter, R. J., Pasternack, G. R., Pardoll, D. M. & Jaffee, E. M. (1995) *Anal. Biochem.* **226**, 15-25.
- den Haan, J. M., Sherman, N. E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J. W., Skipper, J., Shabanowitz, J., Hunt, D. F., Engelhard, V. H. & Els Goulmy (1995) *Science* **268**, 1476-1480.
- Sykulev, Y., Brunmark, A., Tsomides, T. J., Kageyama, S., Jackson, M., Peterson, P. A. & Eisen, H. N. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 11487-11491.
- Iezzi, G., Protti, M. P., Rugarli, C. & Bellone, M. (1996) *Cancer Res.* **56**, 11-15.
- Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B. & Frost, P. (1990) *Cell* **60**, 397-403.
- Hunt, D. F., Yates, J. R. d., Shabanowitz, J., Winston, S. & Hauer, C. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6233-6237.
- Lenz, J., Crowther, R., Straceski, A. & Haseltine, W. (1982) *J. Virol.* **42**, 519-529.
- Hayashi, H., Matsubara, H., Yokota, T., Kuwabara, I., Kanno, M., Koseki, H., Isono, K., Asano, T. & Taniguchi, M. (1992) *J. Immunol.* **149**, 1223-1229.
- Horowitz, J. M. & Risser, R. (1985) *J. Virol.* **56**, 798-806.
- Klarnet, J. P., Matis, L. A., Kern, D. E., Mizuno, M. T., Peace, D. J., Thompson, J. A., Greenberg, P. D. & Cheever, M. A. (1987) *J. Immunol.* **138**, 4012-4017.
- Matis, L. A., Shu, S., Groves, E. S., Zinn, S., Chou, T., Kruisbeek, A. M., Rosenstein, M. & Rosenberg, S. A. (1986) *J. Immunol.* **136**, 3496-501.
- Corr, M., Boyd, L. F., Frankel, S. R., Kozlowski, S., Padlan, E. A. & Margulies, D. H. (1992) *J. Exp. Med.* **176**, 1681-1692.
- Schulz, M., Aichele, P., Schneider, R., Hansen, T. H., Zinkernagel, R. M. & Hengartner, H. (1991) *Eur. J. Immunol.* **21**, 1181-1185.
- Risser, R., Horowitz, J. M. & McCubrey, J. (1983) *Annu. Rev. Genet.* **17**, 85-121.
- O'Donnell, P. V., Stockert, E., Obata, Y. & Old, L. J. (1981) *Virology* **112**, 548-563.
- Hartley, J. W., Wolford, N. K., Old, L. J. & Rowe, W. P. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 789-792.
- Nowinski, R. C., Hays, E. F., Doyle, T., Linkhart, S., Medeiros, E. & Pickering, R. (1977) *Virology* **81**, 363-370.
- Timmons, P. M., Brickell, P. M., Latchman, D. S. & Rigby, P. W. (1991) *Nucleic Acids Res.* **19**, 7215-7218.
- DeLeo, A. B., Shiku, H., Takahashi, T., John, M. & Old, L. J. (1977) *J. Exp. Med.* **146**, 720-734.
- Chen, Y.-T., Obata, Y., Stockert, E. & Old, L. J. (1985) *J. Exp. Med.* **162**, 1134-1148.
- DeLeo, A. B., Chang, K. S., Wivel, N. A., Appella, E., Old, L. J. & Law, L. W. (1982) *Int. J. Cancer* **29**, 687-693.
- Sikora, K., Koch, G., Brenner, S. & Lennox, E. (1979) *Br. J. Cancer* **40**, 831-838.
- Lennox, E. S., Lowe, A. D., Cohn, J. & Evan, G. (1981) *Transplant. Proc.* **13**, 1759-1761.
- Bookman, M. A., Swerdlow, R. & Matis, L. A. (1987) *J. Immunol.* **139**, 3166-3170.
- Matis, L. A., Ruscetti, S. K., Longo, D. L., Jacobson, S., Brown, E. J., Zinn, S. & Kruisbeek, A. M. (1985) *J. Immunol.* **135**, 703-713.
- Ono, M., Kawakami, M. & Takezawa, T. (1987) *Nucleic Acids Res.* **15**, 8725-8737.
- Ono, M. (1986) *J. Virol.* **58**, 937-944.
- Medstrand, P. & Blomberg, J. (1993) *J. Virol.* **67**, 6778-6787.
- Ono, M., Yasunaga, T., Miyata, T. & Ushikubo, H. (1986) *J. Virol.* **60**, 589-598.
- Sauter, M., Schommer, S., Kremmer, E., Remberger, K., Dolken, G., Lemm, I., Buck, M., Best, B., Neumann-Haefelin, D. & Mueller-Lantzsch, N. (1995) *J. Virol.* **69**, 414-421.
- Wahlstrom, T., Suni, J., Nieminen, P., Narvanen, A., Lehtonen, T. & Vaheri, A. (1985) *Lab. Invest.* **53**, 464-469.
- Moyret, C. F., Bernard, D. J., Maurizis, J. C., Chollet, P. & Plagne, R. (1988) *Anticancer Res.* **8**, 1279-1283.
- Moshier, J. A., Luk, G. D. & Huang, R. C. (1986) *Biochem. Biophys. Res. Commun.* **139**, 1071-1077.
- Ono, M., Kawakami, M. & Ushikubo, H. (1987) *J. Virol.* **61**, 2059-2062.
- Faff, O., Murray, A. B., Schmidt, J., Leib-Mosch, C., Erfle, V. & Hehlmann, R. (1992) *J. Gen. Virol.* **73**, 1087-1097.
- Vogetseder, W., Dumfahrt, A., Mayersbach, P., Schonitzer, D. & Dierich, M. P. (1993) *AIDS Res. Hum. Retroviruses* **9**, 687-694.
- Cianciolo, G. J. & Snyderman, R. (1986) *Cancer Metastasis Rev.* **5**, 15-27.
- Slingluff, C. L., Jr., Cox, A. L., Henderson, R. A., Hunt, D. F. & Engelhard, V. H. (1993) *J. Immunol.* **150**, 2955-2963.
- Storkus, W. J., Zeh, H. J. d., Maeurer, M. J., Salter, R. D. & Lotze, M. T. (1993) *J. Immunol.* **151**, 3719-3727.
- Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ildstad, S. T., Martin Kast, W., DeLeo, A. B. & Lotze, M. T. (1995) *Nat. Med.* **1**, 1297-1302.
- Mandelboim, O., Vadai, E., Fridkin, M., Katz-Hillel, A., Feldman, M., Berke, G. & Eisenbach, L. (1995) *Nat. Med.* **1**, 1179-1183.
- Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, L. M., Karasuyama, H., Baker, M. & Pardoll, D. M. (1991) *Science* **254**, 713-716.