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ORIGINAL ARTICLE

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SV40 Tag-specific cytotoxic T lymphocytes generated from the peripheral blood of malignant pleural mesothelioma patients

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Abstract Malignant pleural mesothelioma (MPM) is an aggressive cancer, with survival of less than one year following diagnosis and treatment with current protocols. Recent studies have demonstrated the presence of the simian virus 40 (SV40)-like, large tumor antigen (Tag) in nearly 60% of MPMs. SV40 Tag is a viral-encoded tumor-specific antigen, and thus a potential target for the induction of anti-tumor immunity and the development of therapeutic vaccines. We describe here evidence for the existence of SV40 Tag-specific immune responses in patients with MPM whose tumors express Tag. Humoral immunity was demonstrated by the detection of IgG titers against Tag in serum samples from 1/3 of patients examined. CTLs were generated from the peripheral blood of an HLA-$A2^{+}$ MPM patient with a synthetic peptide representing an HLA-A2 binding epitope in SV40 Tag. The CTLs demonstrated epitope fine specificity, in that other peptides from SV40 Tag and a peptide from influenza virus were not recognized in the context of HLA-A2. Moreover, the CTLs were capable of recognizing mesothelioma tumor cells that expressed SV40 Tag, in an MHC class I restricted manner.

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Introduction

Current estimates indicate that one in three Americans will suffer from cancer in their lifetime and one in six will die of cancer. Malignant pleural mesothelioma (MPM) is an exceptionally lethal form of cancer originating in the serosal lining of the pleura. Survival following diagnosis for advanced stage disease is less than one year. Currently available therapies including surgery, radiation and chemotherapy fail to significantly alter MPM progression and subsequent prognosis [16]. Thus, the need for new alternative cancer therapies for MPM is profoundly important.

Simian virus 40 (SV40) is a member of the papovavirus family, which includes human papilloma virus (HPV) and the human viruses JCV and BKV. SV40 was first discovered as a contaminant in cultures of monkey kidney cells [33]. Early studies demonstrated that SV40 infection was capable of inducing tumors in rodents and transforming multiple cell types of both human and rodent origin in vitro [31, 19]. Evaluation of the cellular transformation events associated with SV40 infection revealed that expression of the viral early T antigen (SV40 Tag) is required to establish and maintain the transformed phenotype [10]. In addition, cell cultures transformed by infection with SV40 express significant amounts of SV40 large tumor antigen (Tag) in the cell nucleus, the cytoplasm and, to a lesser extent, as an intact transmembrane protein on the transformed cell's surface [11]. Expression of Tag in SV40 transformed cells suggests that SV40 Tag may represent a viral-encoded tumor antigen capable of inducing tumor-specific immunity. Indeed, multiple studies have confirmed this hypothesis by demonstrating that mice inoculated with inactivated syngeneic SV40 transformed cells, immunized

with SV40 Tag purified from transformed cells or recombinant SV40 Tag (rTag) elicit SV40 Tag-specific immune responses that protected mice from a subsequent lethal tumor challenge with live syngeneic SV40 transformed cells [1, 15, 35, 30, 38]. Thus, SV40 Tag represents a viral-encoded tumor-specific antigen that is a potent target for the induction of immune responses capable of destroying SV40 transformed cells in vivo.

Recently, SV40-like DNA sequences have been amplified from human tumors of the bone (osteosarcomas), brain (ependymomas and glioblastomas), and pleural lining (malignant pleural mesothelioma). Over 60% of ependymomas, examined by multiple laboratories, contain SV40 DNA and exhibit SV40 Tag protein expression [2]. More recently, a study from China using immunoprecipitation and Western blot analysis, demonstrated the presence of SV40 Tag in 100% of the ependymomas and choroid plexus tumors tested [39]. This group also reported SV40 Tag expression in the majority of the astrocytomas, glioblastomas, and meningiomas tested and could not detect SV40 Tag in any normal brain tissue specimens or non-malignant brain samples [39]. In addition, it has been reported that nearly 60% of MPMs contain SV40 Tag genetic sequences and proteins [14, 22, 32]. The viral genes and gene products though present in MPM tumors could not be found in the non-malignant pleural tissues of the same patient. Further evidence for SV40 association with certain forms of human cancer was recently reported by Butel and co-workers, who recovered infectious virus from a human brain tumor [12]. Sequencing the genome of the viral isolate confirmed that the virus was indeed authentic SV40 and revealed that is was a previously unknown strain of SV40, suggesting that multiple strains of SV40 may be capable of infecting humans.

Tumors with possible viral associations, like MPM, represent ideal candidates for evaluating antigen-specific active vaccination therapies against cancer. Such tumors express viral-encoded, tumor-specific antigens not expressed by normal tissues, suggesting that immune responses could be induced against the antigen that would selectively kill tumors that express the antigen. Though mounting evidence supports the association of SV40 viruses with certain human bone, brain and lung cancers (i.e. MPM), little is known about anti-SV40 immunity in patients that harbor these malignancies. To address this, we evaluated the presence of SV40 Tag-specific immune responses in patients diagnosed with MPM, whose tumors were positive for SV40 Tag expression.

Materials and methods

Clinical samples

After obtaining informed consent, blood specimens were collected from patients undergoing surgical treatment for MPM. Peripheral blood samples were collected in heparin-treated tubes, processed by centrifugation over Histopaque (Sigma) and the peripheral blood mononuclear cells (PBMCs) were isolated and stored in liquid nitrogen prior to use. Serum samples were collected at the same time as PBMCs and stored at –20 °C prior to use. HLA typing was performed on samples of patients' PBMCs. Seventeen patients were HLA typed; six expressed HLA-A2 and eleven expressed various other HLA-A haplotypes, no two alike; therefore, HLA-A2 patients' specimens were selected for the study. Sufficient quantities of serum and PBMCs were available for further study for only three of the HLA-$A2^+$ patients identified, whose tumors were also positive for SV40-like DNA sequences. Serum was not available for the non-HLA-$A2^+$ patients.

Cells lines

All cell lines were maintained in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum, 2 mM L-Gln, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 10 mM HEPES buffer, with incubation at 37 °C in 5% CO_2. Cells were passaged when confluent by incubation with trypsin/EDTA (BioWhittaker). The pleural mesothelioma cell lines, COS-7, and mKSA cell lines were available in our laboratory. We were unsuccessful in generating autologous mesothelioma cell lines; thus we were forced to use the allogeneic cell lines available in our laboratory. The melanoma cell line was generated in the Surgery Branch (NCI, NIH). The HLA-$A2^+$ lymphoblast cell line T2 and the MHC class-I-negative cell line Daudi were purchased from ATCC (Rockville, Md.). Transfer of the gene for SV40 Tag into the HLA-$A2^+$ mesothelioma cell line was accomplished using Lipofectamine Plus (Life Tech., Gaithersburg, Md.) and the G418 selectable Tag expressing plasmid, pSV3neo [8]. Stable transfectants were selected in 1 mg/ml G418 prior to use in cytotoxicity assays. HLA sero-typing was performed on all cell lines to confirm MHC class I antigen expression and HLA-A2 haplotype expression.

Immunoassay for the detection of antibodies to SV40 Tag

To detect the presence of antibodies with specificity for SV40 Tag in the serum of patients with MPM, an ELISA was employed, as previously described [5]. Briefly, 50 μl of purified baculovirus-derived SV40 rTag, at a concentration of 4 μg/ml, was coated onto 96-well flat bottom microtiter plates, overnight at 4 °C. Baculovirus-derived recombinant HBsAg was coated onto 96-well plates in a similar manner, and served as a negative control viral antigen in all ELISA assays. Serum samples were titrated in duplicate by serial twofold dilution, beginning at 1:5, followed by incubation for 1 h at 37 °C. Antibody recognition was detected by incubation with a goat anti-human IgG specific antibody conjugated with horseradish peroxidase (HRP) (Sigma). Non-specific binding was blocked by incubating plates in 10% normal goat serum (NGS) diluted in borate buffered saline (BBS) prior to adding patient serum. All dilutions were done with NGS/BBS. Assays were developed with 2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS) and measured using an automated ELISA-plate reader (Molecular Dynamics) at 405 nm. Serum from cord blood was used as a negative control and sera from mice immunized with SV40 rTag and probed with goat anti-mouse IgG-HRP served as positive controls. Detection of antibodies with reactivity to SV40 Tag peptides that represent B cell epitopes was performed using an ELISA, as previously described by our laboratory [6]. Briefly, peptides were coated onto 96-well flat bottom microtiter plates at a final concentration of 250 ng/ml and incubated overnight at 4 °C. Plates were blocked as described above, then serum was added to duplicate wells at a dilution of 1:5 in 50 μl, followed by 1 h incubation at 37 °C. Antibody binding was detected as described above. Cord serum served as a negative control for antibody and an irrelevant peptide from gp120 of HIV-1 (designated 5077) served as a control for peptide specificity [6].

Synthetic peptides

SV40 Tag peptides were selected, based on predicted HLA-A2.1 binding motifs [21]. All synthetic peptides used for CTL generation and cytotoxicity assays were determined to be >95% pure by HPLC and of a single 9 amino acid species by mass spectroscopy analysis (AnaSpec, Inc., San Jose, Calif.). Peptides were reconstituted in DMSO and stored at a concentration of 2 mg/ml at –20 °C prior to use (Table 2).

Generation of cytotoxic T cells

To generate cytotoxic T cells, PBMCs were cultured in 24-well plates at 2×10^6 cells/well with 5 μg/ml of peptide in the presence of 100 U/ml IL-4 and 200 U/ml GM-CSF, using previously published methods [24, 27]. IL-2 (150 IU/ml) and IL-12 (5 ng/ml) (PeproTech, Inc., N.J.) were added on day 5 and replaced every 2–3 days, as needed to support T cell growth. T cell cultures were restimulated every 14 days with irradiated, allogeneic HLA-$A2^+$ EBV-B cell lines (alternated in an attempt to avoid the generation of responses against unshared allo-antigens) that had been pulsed overnight at 37 °C with 5 μg/ ml peptide. Prior to assay, at 6 weeks of culture (day 42) and following 4 restimulations with antigen, T cell cultures were determined to be >90% $CD8^+$ by flow-cytometry. Medium for culture of T cells consisted of RPMI-1640 supplemented with 10% heat-inactivated human AB serum, 2 mM L-Gln, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 10 mM HEPES buffer, with incubation at 37 °C in 5% CO_2.

Cytotoxicity assay

Cytotoxic T cell activity and specificity was assessed using a standard 4-h chromium release assay. Briefly, target cells were labeled with ^{51}Cr, then combined with $CD8^+$ T cells at varying effector to target ratios (E:T). Supernatants were harvested using a Skatron cell harvester and radioactivity was measured on a standard gamma counter. Percent specific lysis was calculated for the mean of triplicate determinations using the following equation:

$$[(E - S)/(M - S)] \times 100$$

where E = experimental release as a result of lysis in the presence of T cells, S = spontaneous release of radioactivity, and M = maximum release by incubation of targets with 2% SDS. Targets consisted of HLA-$A2^+$ or -$A2^-$ tumor cells lines that expressed or did not express SV40 Tag. For determining peptide epitope specificity, targets were HLA-$A2^+$ T2 cells alone or T2 cells pulsed with 5 μg/ml of peptide for 1 h at 37 °C.

Western blot analysis

To confirm SV40 Tag expression in transfectants compared to controls, Western blot analysis was performed on whole cell lysates, as described previously [7]. Briefly, 10^7 cells were washed three times with PBS and lysed with 0.5 ml of 50 mM Tris-HCl buffer containing 100 mM NaCl, 100 μM aprotinin and leupeptin, and 1% (v/v) NP-40 (pH 9.0). Following clarification by high-speed centrifugation, 100 μl aliquots of the final supernatants were resolved on an SDS-PAGE gel (12%), then electrotransferred to a nitrocellulose membrane. The presence of SV40 Tag in the lysates was detected using high-titer murine anti-SV40 Tag serum collected from mice immunized with SV40 rTag and a goat anti-mouse IgG specific antibody conjugated to HRP as the secondary antibody (Sigma). The murine anti-serum was extensively characterized for use in Western blots in our laboratory [7], and found to be more sensitive and reliable than commercially available monoclonal reagents. Blots were developed using the peroxidase sensitive substrate 3,3'-di-aminobenzidine (DAB) (Sigma).

RNA isolation and RT-PCR

RNA was isolated from confluent tumor cell monolayers with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. The integrity of the RNA was determined by electrophoresis on 2% agarose gels and ethidium bromide staining. RNA was quantified by UV absorption and stored at a concentration of 1 μg/ml at –70 °C. Prior to cDNA generation, all RNA samples were treated with RNAse-free DNAse (Promega) to remove residual contaminating DNA and adjusted to 1 μg/ml. After reverse transcription of 1 μg of DNA-free RNA, using oligo (dT) priming and superscript II reverse transcriptase (Life Technologies), the cDNA was amplified using a Taq PCR core kit (Qiagen) in a total volume of 50 μl and 0.5 U Taq per reaction. Primers for the detection of SV40 Tag were: (sense) 5′-CAAGCAACTCCAGCATC-3′ and (anti-sense) 5′-CCAGAAGAAGCAGAGGAAAC-3′. Primers for the detection of housekeeping genes were: β-actin for human (XAHR17 and XAHR20, Research Genetics) and GAPDH for mouse, (sense) 5′-CCGGTGCTGAGTATGTGG-3′ and (anti-sense) 5′-TCTGGGTGGCAGTGATGG-3′. PCR conditions were an initial denaturation of 3 min at 94 °C, followed by 1 min denaturation at 94 °C, annealing for 1 min, extension for 1 min at 72 °C for 30 cycles, ending with a 5 min extension at 72 °C. Annealing temperatures were 61 °C for SV40 Tag primers, 60 °C for GAPDH and 55 °C for β-actin. All annealing temperatures were determined empirically using a Biometra gradient thermocycler. Ten-microliter aliquots of amplification products were visualized on 2% agarose gels with ethidium bromide staining. Reactions substituting H_2O for cDNA template were included for each primer set as negative controls.

Flow cytometry

Tumor cells were stained with the monoclonal antibody W6/32 (anti-HLA-A, B, C) or an isotype matched control. To enhance surface expression of MHC-I molecules, tumor cells were cultured in the presence of IFN-γ (500 U/ml) for 72 h prior to flow cytometric analysis.

Results

Serologic evidence for immunity to SV40 Tag in MPM patients

Archival blood samples from 17 patients diagnosed with MPM were screened for expression of the MHC class I haplotype, HLA-A2.1. Sufficient quantities of serum and PBMCs necessary for the study were obtained from three of six HLA-$A2.1^+$ patients identified. To determine the presence of an immune response to SV40 in the three patients, an ELISA was employed to measure serum antibody titers to SV40 Tag [5]. Using cord blood as a negative control, we established an absorbance of 0.1 (405 nm) at a 1:5 dilution as a standard for positive reactivity with SV40 Tag by ELISA. Cord blood was chosen to represent human normal serum since "pre-immune" serum samples did not exist for these patients. Evaluation of the patient sera demonstrated that only patient number one (patient 1) exhibited reactivity to SV40 Tag by ELISA. At a dilution of 1:5 the absorbance (405 nm) for serum from patient 1 was 0.22, where as the absorbance for patients 2 and 3 at a similar dilution were below the established standard of 0.10 (0.08 and 0.04, respectively) (Fig. 1). Twofold dilutions of serum from

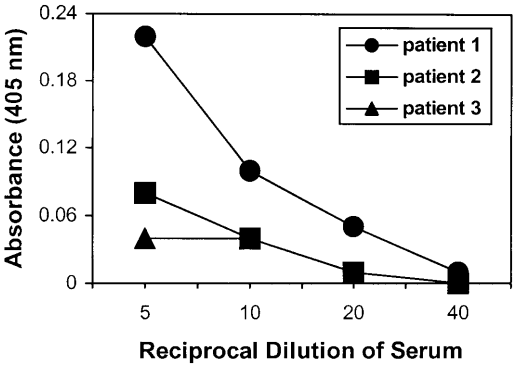


Fig. 1 Anti-SV40 Tag IgG titers in the serum of patients with MPM. Serum samples from three HLA-A2$^+$ patients were assayed for reactivity against SV40 Tag by ELISA. Individual serum samples were titrated be serial twofold dilutions, beginning with 1:5, on microtiter plates coated with rTag. Shown are the mean absorbance values for the IgG reactivity of duplicates for each dilution. Errors were negligible

patient 1 (beginning with 1:5) demonstrated a decrease in Tag reactivity with increasing dilution, ending with no detectable antibodies at 1:40 (Fig. 1). These results indicate low, but detectable, IgG antibody titers to Tag. None of the sera recognized rHBsAg on the solid phase in similar assays (data not shown). These data indicate that at least one of the three patients appeared to have generated an immune response against SV40 Tag. To confirm that the antibody reactivity was not due to cross-reactivity with Tags from two human related viruses, BKV and JCV, serum from patient 1 was tested for recognition of a segment of the carboxyl terminus of Tag unique to SV40 Tag. Serum from patient 1 recognized a 19-mer peptide corresponding to amino acid residues 690–708 from the carboxyl-terminus of SV40 Tag [6], and failed to bind a control peptide from HIV gp120 (Table 1). This result demonstrates that the antibody response was specific for the Tag of SV40 since the other papovavirus Tags (BKV and JCV) do not contain the 690–708 peptide.

SV40 Tag peptide induced cytotoxic T lymphocytes

Numerous studies using murine models and human lymphocytes indicate that CD8$^+$ cytotoxic T lymphocytes (CTLs) represent a critical component of cellular immunity involved in solid tumor killing. To examine the CTL responses against SV40 in the three HLA-A2.1 MPM patients, the protein sequence for SV40 Tag was scanned for putative HLA-A2.1 binding motifs [21] and six peptides with the highest predicted affinity for HLA-A2.1 were used to generate CTLs in vitro (Table 2). PBMCs from each patient were cultured with peptide in the presence of IL-4 and GM-CSF to activate resident professional antigen-presenting cells. Cultures were restimulated every 14 days, as described in the methods. By day 49 of culture, following 4 stimulations with peptide, T cell expansion

Table 1 Serum recognition of an SV40 Tag-specific synthetic peptide epitope

Serum (1:5 dilution)	Peptide	
	690–708[a]	5077[b]
Cord blood	0.021[c]	0.028
Patient 1	0.173	0.019
Patient 2	0.012	0.009
Patient 3	0.008	0.011

[a]Nineteen-amino-acid synthetic peptide specific for the carboxy-terminus of SV40 Tag, amino acid positions 690–708
[b]Synthetic peptide from gp120 of HIV-1
[c]Mean OD_{405} for duplicate determinations

was observed for all three patients stimulated with each of the six peptides (total of 18 T cell cultures), and each culture was primarily CD8$^+$ (>90% for each patient), as determined by flow cytometry (data not shown). Peptide-specific CTL-mediated killing was assessed against peptide pulsed T2 cells as targets. MHC class-I-negative Daudi cells were used as a non-specific target. None of the 18 CTL cultures exhibited lytic activity against Daudi cells, demonstrating lack of non-specific killing (not shown). Only one of the six peptides was capable of eliciting CTLs in one of the three patients. CTLs were generated from the PBMCs of patient 1 following stimulation with peptide 2 (VLLLLGMYL). The VLLLLGMYL peptide generated CTLs from patient 1 and demonstrated 85% specific lysis of T2 cells pulsed with peptide VLLLLGMYL compared to T2 cells alone (<10%) or Daudi cells (<10%) (Fig. 2). The VLLLLGMYL peptide failed to induce specific CTLs in either patient 2 or patient 3 (Fig. 2). The CTLs generated from patient 1 were epitope specific, since none of the other five Tag peptides were recognized, nor an HLA-A2 peptide from the M1 protein of influenza (not shown).

Epitope fine specificity of peptide-generated cytotoxic T lymphocytes

CTLs generated from patient 1 by in vitro stimulation with SV40 Tag peptide VLLLLGMYL were examined for epitope fine specificity by assessing recognition of distinct SV40 Tag peptides possessing HLA-A2.1 binding motifs (Table 2) using a standard 4-h ^{51}Cr-release assay. As demonstrated in Fig. 2, neither Daudi cells nor T2 cells alone were lysed by the CTLs, whereas T2 cells pulsed with VLLLLGMYL peptide were lysed (>90% lysis at an E:T ratio of 40:1) (Fig. 3). However, these CTLs failed to lyse T2 cells pulsed with any of the other five SV40 Tag peptides evaluated in this study (Table 2). Percent lysis of T2 cells pulsed with the distinct A2 binding peptides was <10% at all E:T ratios examined (Fig. 3). In addition, these CTLs failed to lyse T2 cells pulsed with an HLA-A2.1 peptide from the matrix protein (M1) of influenza virus (not shown). Thus, the

Table 2 SV40 Tag synthetic peptides with HLA-A2.1 binding motifs

Peptide designation	Sequence	SV40 Tag position	Predicted binding t(1/2) (min)
1	KLCTFSFLI	214–222	805
2	VLLLLGMYL	285–293	739
3	ALTRDPFSV	235–243	403
4	FLTPHRHRV	197–205	320
5	CLLPKMDSV	396–404	290
6	FQYSFEMCL	295–303	121

CTLs generated from the PBMCs of patient 1 by in vitro stimulation with SV40 Tag peptide VLLLLGMYL appeared to be specific for the stimulating peptide in the context of HLA-A2.1.

SV40 Tag-specific tumor cell killing by peptide-generated cytotoxic T lymphocytes

It is important to determine whether CTLs generated by in vitro peptide stimulation are capable of recognizing and lysing tumor cells that express the antigen of interest. This would demonstrate that the tumor cells have processed the antigen and presented the peptide epitope in the context of the appropriate MHC class I molecule, enabling recognition by the CTLs. In order to address this for the CTLs generated by in vitro stimulation of PBMCs with peptide 2 from SV40 Tag, we transfected an HLA-A2.1$^+$ mesothelioma cell line with the gene for SV40 Tag. Expression of mRNA encoding SV40 Tag by the transfected cell line was confirmed by RT-PCR. SV40 Tag expression was not detected in either an untransfected HLA-A1$^+$ mesothelioma cell line or a melanoma cell line, nor was Tag expression detected in the HLA-A2.1$^+$ mesothelioma cell line prior to transfection with SV40 Tag cDNA (Fig. 4B). Protein expression of SV40 Tag was confirmed by Western blot analysis. A band of approximately 90 kDa [7] was observed for the transfected mesothelioma cell line (Fig. 4A, *lane 3*), as well as the murine mKSA cells (Fig. 4A, *lane 1*) and COS7 monkey cells (Fig. 4A, *lane 5*) that were generated by infection with SV40 and were positive for expression of SV40 Tag when compared to staining of rSV40 Tag (1 μg) (Fig. 4A, *lane 6*). Examination of the mesothelioma cell line prior to SV40 Tag transfection revealed no detectable levels of SV40 Tag protein expression by Western blot (Fig. 4A, *lane 4*). Next, these tumor cell lines were employed to measure the antigen-specific tumor killing capacity of the Tag-peptide generated CTLs. SV40 Tag-peptide-specific CTLs from patient 1 were capable of recognizing and lysing the HLA-A2.1$^+$ mesothelioma cell line that expressed SV40 Tag following transfection with pSV3neo, an SV40 Tag expression vector (percent lysis of approximately 15% at an E:T ratio of 10:1) (Fig. 5). However, the HLA-A2$^+$ mesothelioma cell line was not recognized when transfected with a Tag-negative control vector, pSV2neo (lysis $< 5\%$). In addition, neither an HLA-A2$^+$/SV40 Tag$^-$ melanoma cell line, nor an HLA-A2$^-$/SV40 Tag$^+$ mesothelioma cell line were recognized ($< 5\%$ lysis) (Fig. 5). Unfortunately, we were unable to test lysis of targets at higher E:T ratios due to the limited number of CTLs available at day 49 of culture. CTLs generated by in vitro stimulation of PBMCs from patient 1 with peptide 2 (VLLLLGMYL), demonstrated SV40 Tag-specific tumor killing in vitro. MHC class-I-restricted killing by the peptide-generated CTLs was confirmed by blocking of tumor lysis with a mAb that was specific for MHC class I (W6/32), but not by either an anti-MHC class II mAb (IVA12) or an irrelevant mAb (not shown). Although a 3-fold increase in lytic activity was observed for the HLA-A2$^+$/SV40 Tag$^+$ mesothelioma cell line, 15% lysis seemed low. To examine this we measured MHC-I expression on the tumor target by flow cytometry and observed very low levels of MHC-I expression, which were only marginally increased in the presence of IFN-γ (Fig. 6). The low level of MHC-I expression and the low effector to target cell ratio (10:1) likely accounts for the minimal lysis of the tumor target shown in Fig. 5. Nonetheless, these data suggest that human mesothelioma cells are capable of processing and presenting the HLA-A2 binding SV40 Tag peptide VLLLLGMYL, and that CTLs specific for the peptide are capable of lysing SV40 Tag-expressing mesothelioma cells in an HLA-A2 restricted manner.

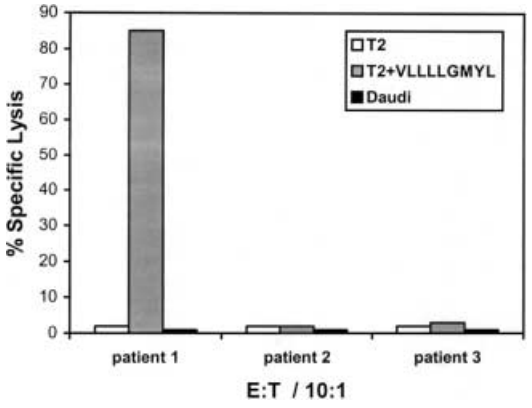


Fig. 2 Generation of CTLs from the peripheral blood of MPM patients by in vitro stimulation with synthetic peptides. A panel of six synthetic peptides representing HLA-A2 binding epitopes in SV40 Tag were incubated with patients' PBMCs to generate CTLs. Peptide reactivity was measured by lysis of T2 cells +/– peptide. Daudi served as a control for non-specific lysis. Killing of T2 cells pulsed with SV40 Tag peptide 2 (VLLLLGMYL) by CTLs from three individual patients. Shown are the mean values for triplicate determinations

Discussion

The purpose of this study was to determine if patients diagnosed with MPM, whose tumors express SV40 Tag,

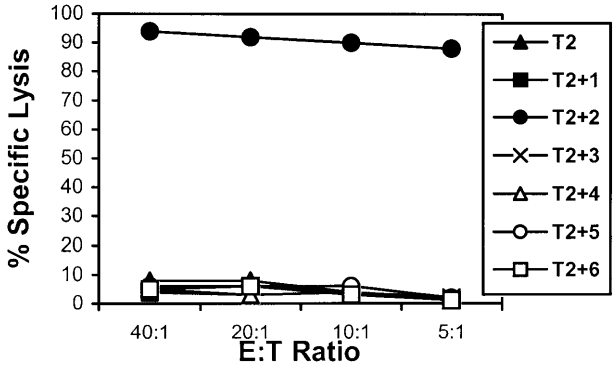


Fig. 3 Epitope specificity of CTLs generated by stimulation with Tag peptide 2 (VLLLLGMYL). CTLs generated from patient 1 PBMCs following stimulation with Tag peptide 2 were examined for reactivity against five distinct HLA-A2 binding SV40 Tag peptides pulsed onto T2 cells. T2 cells without peptide served as a control for specific lysis. Shown are the mean values for triplicate determinations

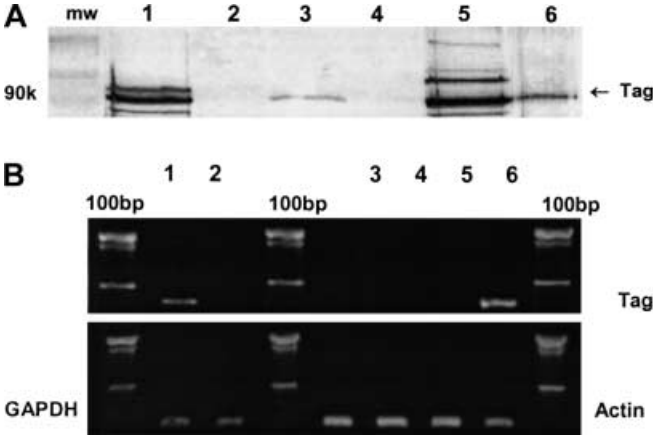


Fig. 4A, B Expression of SV40 Tag in tumor cell lines. **A** Western blot analysis of SV40 Tag protein expression in tumor cell lines. A 90 kDa band was observed for SV40 transformed mKSA murine tumor cells and COS-7 cells (*lanes 1 and 5*, respectively), as well as the HLA-A2 mesothelioma tumor cell line transfected with SV40 Tag (*lane 3*), following incubation with murine anti-Tag serum. No Tag was detected in an HLA-A2 melanoma cell line or the HLA-A2 mesothelioma cell line prior to transfection (*lanes 2 and 4*, respectively). Recombinant SV40 Tag (1 μg) served as a size reference control (*lane 6*). **B** RT-PCR analysis of SV40 Tag mRNA expression in tumor cell lines. A 400 bp RT-PCR product confirming SV40 Tag expression was observed in mKSA SV40 transformed murine tumor cells and the Tag transfected HLA-A2 mesothelioma cell line (*lanes 1 and 6*, respectively). Tag mRNA expression was not detected in murine 3T3 cells (negative control), HLA-A1 mesothelioma cell line, an HLA-A2 melanoma cell line, or the HLA-A2 mesothelioma cell line prior to transfection (*lanes 2, 3, 4 and 5*, respectively). Actin and GAPDH served as controls for gene expression (human and mouse, respectively)

provide evidence of an immune response against SV40 Tag, thereby rendering Tag a potential target for active immunotherapy of MPM in humans.

Studies have demonstrated that detection of serum IgG antibodies with tumor antigen specificity is indicative of the presence of anti-tumor immunity involving tumor-reactive T cells, as well as antibody-producing B cells. Previously, Jager et al. demonstrated a correlation between the presence of serum antibodies with specificity for the tumor-associated NY-ESO-1 and the ability to generate CTLs against NY-ESO-1 in the same patient [18]. Observations such as this are the premise for the recent development of a novel approach to clone tumor antigens from human malignancies, including melanoma and colon cancer. The technique, termed serological analysis of recombinant cDNA expression libraries (SEREX) [20, 28, 18], employs patients' sera and autologous tumor to identify tumor antigens such as NY-ESO-1, thus demonstrating the usefulness of anti-tumor antibodies as an indication of existing immune responses to tumor antigens in cancer patients. Interestingly, when sera from patients diagnosed with MPM whose tumors tested positive for expression of SV40 Tag were examined for the presence of anti-Tag antibody titers, 1/3 demonstrated an anti-Tag IgG response by ELISA (Fig. 1). Further, this antibody response was confirmed to be SV40 Tag-specific by testing for recognition of a peptide unique to the carboxy-terminus of SV40 Tag (Table 1). Possible explanations for the absence of Tag recognition by sera from 2/3 MPM patients may be tolerance mechanisms employed as the tumor developed (MPM is a particularly slow growing malignancy) or simply waning of an initial immune response over time. Nonetheless, 1/3 MPM patients demonstrated a detectable serum antibody response to SV40 Tag, suggesting that anti-Tag T cells may also exist in patients with MPM.

Numerous studies have demonstrated that T lymphocytes represent an important component of anti-tumor immune responses involved in solid tumor killing. The vast majority of tumor reactive T cells that have been defined for humans are $CD8^+$ MHC class-I-restricted CTLs [4, 25]. $CD8^+$ CTLs recognize peptide fragments (8–10 amino acids in length) of intracellular protein antigens in the context of MHC class I molecule heavy chains in association with β-2 microglobulin. These tri-molecular complexes are expressed on the tumor cell surface, providing extracellular presentation of endogenously expressed and enzymatically processed protein fragments (peptides) of tumor antigen proteins for a CTL to engage using its antigen-specific receptors (TCR). Herein, we demonstrate the generation of CTLs from the PBMCs of 1/3 MPM patients with specificity for an HLA-A2 binding peptide epitope from SV40 Tag (Fig. 2). CTLs were generated by in vitro stimulation of PBMCs with synthetic peptides representing putative HLA-A2 binding epitopes from SV40 Tag (Table 2). These CTLs were peptide/epitope- specific (Fig. 3) and capable of killing HLA-A2 mesothelioma cells that expressed SV40 Tag (Fig. 5). The recognition of SV40 Tag-expressing MPM tumor cells by peptide-specific CTLs in an HLA-A2-restricted fashion was demonstrated by transfecting the gene for SV40 Tag into an HLA-A2 MPM tumor cell line (Fig. 4A, B). Unfortunately, tumor cell lines do not exist for any of the MPM

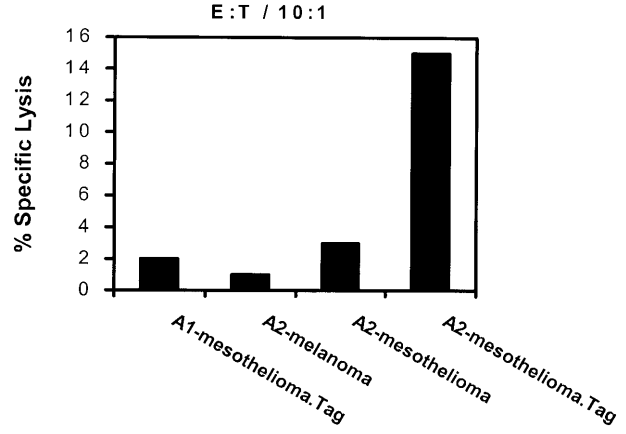


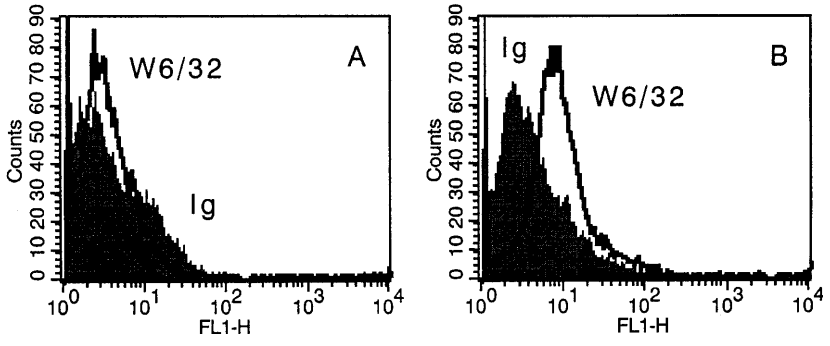
Fig. 5 Antigen-specific tumor cell killing by peptide-generated CTLs. CTLs generated from patient 1 PBMCs following stimulation with Tag peptide 2 (VLLLLGMYL) were examined for reactivity against targets that were: Tag-positive and HLA-A2-negative; Tag-negative and HLA-A2-positive; or Tag-positive and HLA-A2-positive. CTL mediated lysis of SV40 Tag-negative targets; HLA-A1 mesothelioma that expresses Tag (A1-mesothelioma-Tag), HLA-A2 melanoma that is Tag-negative (A2-melanoma), HLA-A2 mesothelioma mock-transfected with the Tag-negative vector, pSV2neo (A2-mesothelioma). Lysis of HLA-A2 mesothelioma cell line post transfection with pSV3neo, an SV40 Tag expression vector (A2-mesothelioma-Tag). Shown are the mean values for triplicate determinations

patients studied, nor is there serum or PBMCs for the MPM patient from whom the HLA-A2 tumor cell line was derived. However, the peptide-specific CTLs only recognized HLA-A2 tumor cells that also expressed SV40 Tag (Fig. 5). Of note, CTLs could only be generated from the patient that also possessed an IgG antibody titer to SV40 Tag. This suggests that, like NY-ESO-1 [18], the presence of an antibody response to SV40 Tag is indicative of a CTL response. To confirm this, the observation would need to be repeated with multiple patients.

Fig. 6A, B Surface expression of MHC class I molecules on mesothelioma cells. **A** Tumor cells expressed minimal amount of MHC class I molecules (*open graph*, staining with mAb W6/32) as determined by flow cytometry. **B** After exposure to 500 U/ml IFN-γ for 3 days, MHC class I expression was only slightly enhanced (*open graph*, staining with mAb W6/32). Control Ig is shown as solid graphs

In addition, it is possible that the panel of peptides examined does not cover all the possible SV40 Tag/HLA-A2 epitopes that may be recognized by CTLs from 2/3 of the MPM patients' PBMCs. For this study, we focused on native peptide epitopes from SV40 Tag with predicted HLA-A2 high binding affinities. The possibility exists that native peptides with lower predicted affinities, which could be modified to increase binding to HLA-A2 by changing amino acid residues in anchor positions to residues that represent high affinity consensus sequences, could have induced CTLs from the non-responding MPM patients PBMCs. Indeed, we have employed the approach of changing anchor residues in peptide epitopes to increase MHC class I binding affinity when evaluating a novel candidate tumor antigen from human prostate cancer. We were able to generate CTLs from prostate cancer patients' PBMCs by in vitro stimulation with "heteroclitic peptides" [17] that killed antigen-positive prostate tumor cells in an MHC class-I-restricted manner (unpublished observation). Alternatively, it is possible that immune suppressing or tolerizing mechanism(s) exist in the non-responding MPM patients that precluded them from generating detectable immune responses to SV40 Tag.

The identification of synthetic peptides representing selected immunogenic/immunodominant epitopes from candidate tumor antigens has made possible the generation of human tumor-specific CTLs for other malignancies. For example, repeated exposure of PBMCs from melanoma patients to an immunodominant peptide (up to 6 in vitro restimulations is typical) from the shared melanoma antigen MART-1 resulted in the generation of CTLs that were 50–100 times more potent in specific tumor killing than activated infiltrating lymphocytes harvested from the patient's own tumor [24]. A separate study demonstrated the generation of CTLs with specificity for the shared human melanoma antigen gp100 after 6 weeks in culture by weekly in vitro stimulation of patients' PBMCs with synthetic peptides representing CTL epitopes on gp100 [27]. Of note are the results from a clinical trial involving peptide epitopes from gp100 as a therapeutic vaccine administered to patients with advanced metastatic melanoma. Therapeutic peptide vaccination with IL-2 administration resulted in a higher percentage of clinical responses in melanoma patients than has been seen previously for



therapy of melanoma with IL-2 alone [26]. Although studies that employ CTL peptide epitopes as cancer vaccines are promising, it remains unclear whether tumor-associated antigens that are non-mutated self-tissue differentiation antigens represent viable candidates for active, therapeutic vaccination against human malignancies. Nonetheless, in vitro methods of repeated stimulation (up to six times at weekly or bi-monthly intervals) of T cells isolated from the PBMCs of cancer patients with peptides has become a routine and generally accepted method for identifying candidate tumor-associated antigens that are recognized by T cells.

Tumors with viral associations, such as HPV with cervical carcinoma, represent ideal candidates for evaluating antigen-specific active vaccination therapies against cancer [23, 36]. Such tumors express viral-encoded, tumor-specific antigens not expressed by normal tissues, suggesting that immune responses could be actively induced that would selectively and exclusively kill tumors that express the viral antigen. It has been established that immunization with SV40 Tag induces tumor-specific immunity, which results in protection from a lethal tumor that expresses SV40 Tag in a murine abdominal mesothelioma/SV40 tumor model [9]. Furthermore, Sanda and colleagues have generated a recombinant vaccinia virus vaccine that contains a form of the SV40 Tag gene, which is missing the oncogenic regions of SV40 Tag, which binds and inactivates the Rb and p53 proteins. This Vacc-Tag vaccine induced SV40 Tag-specific immune responses that eliminated existing SV40 Tag expressing tumors in mice [37]. Interestingly, this safety modified Vacc-Tag vaccine (Vacc-mTag) contains the peptide epitope VLLLLGMYL described in this study (Table 2). Further evidence to support the importance of this peptide epitope in HLA-A2-restricted responses to SV40 Tag is the recent report by Tevethia and coworkers. This group immunized C57BL/6 HLA-A2.1 transgenic mice with SV40 Tag and demonstrated the induction of HLA-A2-restricted CTLs that recognized the peptide KCDDVLLL, which overlaps with our HLA-A2 peptide epitope reported here [29]. Of note, Velders et al. recently reported the generation of SV40 Tag-specific CTLs following in vitro stimulation of normal donor PBMCs with HLA-A2 Tag peptides [40]. They found that a peptide corresponding to our peptide 5 (CLLPKMDSV) generated CTLs in one donor, while peptide LMLIWYRPV was capable of generating CTLs in both HLA-$A2^+$ donors tested. Interestingly, neither of these peptides was capable of generating CTLs from the PBMCs of the mesothelioma patients we studied, and our peptide 2 (VLLLLGMYL), which generated CTLs from patient 1 was not capable of raising CTLs in their normal donors [40]. This disparity between SV40 Tag peptides recognized by normal donors and mesothelioma patients may simply be due to differences in T cell repertoires among the individuals examined. However, it is interesting to speculate that perhaps there could be a shift in epitope specificity in normal individuals compared to patients with mesothelioma. If so, this could have an impact on the development of future Tag-peptide-based vaccines to treat mesothelioma patients whose tumors express SV40 Tag.

Though much is known about the oncogenic and immunogenic capacity of SV40 and SV40-induced tumors, it remains unclear whether SV40 is an etiologic agent for certain human cancers [13]. However, recent evidence strongly supports the association of SV40 with MPM formation in humans [34, 3]. Reports such as these and others provide impetus for further study into the role of SV40 and cancer, as well as the development of SV40-targeted immunotherapies against cancers with confirmed SV40 associations. The twofold premise for the development of cancer immunotherapies is that there are differences between tumor cells and normal cells that are manifested as abnormally expressed tumor-associated or tumor-specific antigens, which represent targets for the immune system. And, that cancer patients possess immune cells capable of recognizing these defined tumor antigens, resulting in immune cell-mediated tumor killing. In this study we report evidence for SV40 Tag-specific immunity in patients diagnosed with MPM, a rare but lethal form of cancer containing SV40 Tag, in nearly 60% of cases examined worldwide.

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