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Induction of Tumor Immunity and Cytotoxic T Lymphocyte Responses Using Dendritic Cells Transfected with Messenger RNA Amplified from Tumor Cells[1]

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ABSTRACT

Unique patient-specific tumor antigens may constitute the dominant antigens in the antitumor immune response. Hence, vaccination with the patient's own repertoire of tumor antigens may offer a superior strategy to elicit protective immunity. We have shown previously that dendritic cells transfected with mRNA isolated from tumor cells stimulate potent CTL responses and engender protective immunity in tumor-bearing mice. In the current study, we demonstrate that tumor mRNA, isolated from murine tumor cell lines or from primary human tumor cells microdissected from frozen tissue sections, can be amplified without loss of function. This study provides the foundations for an effective and broadly applicable treatment that does not require the characterization of the relevant antigenic profile in each patient and will not be limited by tumor tissue availability for antigen preparation.

INTRODUCTION

CTL responses are an important effector arm of the antitumor immune response (1). Hence, vaccination with antigens recognized by tumor-specific CTLs may represent an effective strategy for cancer immunotherapy. At present, identification of tumor antigens encoding CTL epitopes have been limited to a few cancers, notably melanoma, reflecting the technical difficulties in obtaining tumor tissue and generating CTL responses from many cancers (2–4). It is not clear which of the known tumor antigens, isolated by virtue of the fact that they were recognized by CTLs from cancer patients, will be effective at inducing protective immunity in the patient. For example, clonal and frequency analysis of CTLs from melanoma patients has revealed that the majority of CTLs were directed against novel antigens, suggesting that the dominant and presumably effective antitumor response is not directed against the current list of known antigens, such as gp100, MART-1, tyrosinase, or MAGE-3 (5).

Vaccination with tumor-derived antigenic mixtures obviates the need to know the identity of the relevant tumor antigens in each patient and will induce a polyclonal immune response against multiple targets on the tumor cell. It has been argued that the dominant antigens in the antitumor response are unique patient-specific tumor antigens, arisen by mutations incidental to the transformation process (6). Thus, vaccination with antigens derived from autologous tumor, which would require the isolation of antigen from each cancer patient, may be superior to using a common source of antigens that could be obtained from allogeneic tumor cell lines. In animal models, vaccination of tumor-bearing mice using DCs loaded with autologous tumor-derived antigens in the form of tumor lysates (7, 8), peptides (9, 10), heat shock proteins (11), or mRNA (12, 13) was highly effective. The scope of vaccination with autologous tumor antigens is, however, limited by the availability of sufficient tumor tissue for antigen preparation, especially because it has been argued that continuous boosting will be required to maintain a protective antitumor immune response (14, 15).

Thus, many cancer patients may not benefit from current strategies of cancer vaccination because an effective tumor antigen associated with their cancer has not yet been identified or because sufficient tumor tissue cannot be obtained for antigen preparation.

We have shown previously that murine and human DCs[3] transfected with mRNA can stimulate potent CTL responses *in vitro* and *in vivo* (12, 16), and treatment of tumor-bearing mice with dendritic cells (DC) transfected with tumor RNA led to a significant reduction in metastases (12) or survival benefit (13). A key advantage of using antigen in the form of RNA is that RNA can be amplified from a few cells, and hence, sufficient, possibly unlimited, amounts of antigen can be generated from a limited amount of tumor tissue (17). Because tumor cells can be isolated to a high degree of purity by microdissection from pathology slides, sufficient antigen could be generated from every cancer patient.

The purpose of this study was to determine whether RNA can be amplified from tumor cells with no loss of biological function. We show that murine DCs transfected with RNA amplified from the melanoma B16/F10.9 cell line are capable of priming CTL responses in mice and engender protective immunity in a postsurgical metastasis setting. Importantly, of added relevance to a clinical setting is the finding that human DCs transfected with RNA amplified from tumor cells microdissected from frozen tissue sections are capable of stimulating primary CTL responses *in vitro*.

This study provides the foundation for a potentially effective and broadly applicable treatment for cancer patients that does not require the characterization of the relevant antigenic profile in each patient and will not be limited by tumor tissue availability for antigen preparation.

MATERIALS AND METHODS

Mice

C57BL/6 mice (H-2^b), 7–8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). In conducting the research described in this report, we adhered to the Guide for the Care and Use of Laboratory Animals, as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Cell Lines

The B16/F10.9 melanoma clone of C57BL/6 origin is a highly metastatic, poorly immunogenic, and a low class I-expressing cell line (18). EL4 cells were established from a thymoma isolated from C57BL/6 mice (ATCC# TIB39). Cells were maintained in DMEM supplemented with 10% FCS, 25

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[3] The abbreviations used are: DC, dendritic cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; PBMC, peripheral blood mononuclear cell; IL, interleukin; CEA, carcinoembryonic antigen; GFP, green fluorescent protein; RT, reverse transcriptase.

mM HEPES, 2 mM L-glutamine, and 1 mM sodium pyruvate. A cell line derived by transfection of B16/F10.9 cells with a murine GM-CSF cDNA plasmid, F10.9/GM, was used as a source of GM-CSF for generating murine DCs. F10.9/GM cells were grown in RPMI 1640 supplemented with 5% FCS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M β-mercaptoethanol and 10 mM HEPES. The human cell lines, SW1463 (CEA^+, HLA-A2 rectum adenocarcinoma) and KLEB (CEA^-, HLA-A2), were obtained from Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, MD). Human cell lines were maintained in DMEM-F12 medium supplemented with 10% FCS, 25 mM HEPES, 2 mM L-glutamine, 1 μg/ml insulin, and 1 mM sodium pyruvate.

Preparation of DCs

Murine. DCs were generated from bone marrow progenitors as described previously (19). Bone marrow from tibias and femurs of C57BL/6 mice were harvested, and the precursors were isolated. The precursors were treated with ammonium chloride Tris buffer for 3 min at 37°C to deplete the RBCs. GM-CSF-containing supernatant was harvested after 24 h from F10.9/GM cells and used at a final dilution of 1:10. Cells were plated at 10^6/ml in GM-CSF-containing medium and incubated at 37°C and 5% CO_2. Three days later, the floating cells (mostly granulocytes) were removed, and the adherent cells were replenished with fresh GM-CSF-containing medium. Four days later, the nonadherent cells were harvested (immature day 7 DCs), washed, and replated at 10^6/ml in GM-CSF-containing medium. After 2–3 days, the nonadherent cells were harvested as DCs (mature day 9 or day 10 DCs), washed, and pulsed with antigen.

Human. DCs were generated from PBMCs, as described previously (20). PBMCs were suspended at $6–7 \times 10^6$/ml in 30 ml of AIM V medium and transferred to tissue culture flasks for adherence. After 1 h of adherence, the nonadherent cells were removed, and 30 ml of AIM V medium with 800 units/ml GM-CSF and 500 units/ml IL-4 were added to the adherent cells. After 7 days, the DCs were harvested and pulsed with antigen. DCs were cryopreserved in 90% autologous plasma at 5×10^6/ml, and PBMCs to be used as responders were cryopreserved at 5×10^7/ml.

Preparation of CEA and GFP RNA by *in Vitro* Transcription

Cloning of pGEM4Z/CEA/A64. Oligonucleotides containing 64 A-T bp, followed by an *Spe*I restriction site, were placed between the *Eco*RI and *Nar*I sites of pGEM4Z (Promega Corp., Madison, WI) to create the plasmid pGEM4Z/A64. A 2467-bp *Sma*I fragment isolated from pGEM3Z-CEA (provided by Jeffrey Schlom) was ligated into the *Sma*I site of pGEM4Z/A64 to create pGEM4Z/CEA/A64. Linearization with *Spe*I, followed by *in vitro* transcription with T7 RNA polymerase (Ambion mMessage mMachine kit, Austin, TX), yields a transcript that contains 47 nucleotides of vector-derived sequence, 85 nucleotides of CEA 5′ untranslated region, 2106 nucleotides corresponding to the coding region of CEA, 265 nucleotides of CEA 3′ untranslated region, 28 nucleotides of polylinker sequence, 64 A nucleotides and 4 nucleotides from the *Spe*I site.

Cloning of pGEM4Z/GFP/A64. The 741-bp *Bam*HI-*Not*I fragment containing the GFP coding region was isolated from pEGFP-N1 (Clontech, Palo Alto, CA). This fragment was cloned into the *Bam*HI and *Nar*I sites of pGEM4Z (Promega), along with oligonucleotides containing a *Not*I half-site, the pGEM4Z polylinker sequence from *Bam*HI to *Eco*RI, 64 A-T bp, an *Spe*I site, and a *Nar*I half-site to create pGEM4Z/GFP/A64. Linearization with *Spe*I, followed by *in vitro* transcription (Ambion mMessage mMachine kit), yields a transcript containing 61 nucleotides of vector-derived sequence, the GFP coding sequence, 24 nucleotides of polylinker sequence, 64 A residues, and 4 nucleotides from the *Spe*I site. RNA was transcribed *in vitro* using T7 RNA polymerase and a cap analogue, as described previously (12).

RT-PCR of Total Tumor RNA

RNA was isolated from tumor cells using the RNeasy kit (Qiagen, Valencia, CA). One μg of total tumor RNA was reverse transcribed in a final volume of 10 μl using SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). First-strand cDNA synthesis was primed with 10 pmol of a modified oligo-dT primer [5′-AAGCAGTGGTATCAACGCAGAGTACT(30)VN-3′], where V is G, A, or C and N is G, A, T, or C. The reaction was incubated at 42°C for 30 min prior to the addition of 10 pmol of the T7 strand switch primer (5′-CTAATACGACTCACTATAGGGCGGG-3′). The reaction was continued for 30 min and was stopped by placing it on ice. The cDNA was amplified by placing 2 μl of the RT reaction into a 100-μl PCR reaction containing 20 pmol of the following primers: T7 PCR (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and 3′ PCR (5′- AAGCAGTGGTATCAACGCAGAGT-3′). Amplification was carried out under standard conditions using Advantage cDNA Polymerase Mix (Clontech Laboratories, Palo Alto, CA). Cycling conditions were as follows: an initial 1 min denaturing step at 95°C, followed by cycling at 95°C for 30 s, 65°C for 30 s, 68°C for 6 min, and a final extension at 68°C for 7 min. The number of cycles performed was dependent on the amount of RNA used in the RT reaction, and for 1 μg of RNA, 18 cycles were typically performed. The amplified cDNA was purified with a QIAquick PCR Purification Kit (Qiagen). The DNA was ethanol precipitated, resuspended, and quantitated by UV spectrophotometry.

In Vitro Transcription of Amplified cDNA

One μg of cDNA was placed in a standard *in vitro* transcription reaction using a T7 mMessage mMachine Kit (Ambion). The reaction was carried out at 37°C for 2–4 h, followed by the addition of DNase I and incubation for 15 min. Ammonium acetate was added, and RNA was isolated by phenol/chloroform extraction and isopropanol precipitation. After centrifugation, the RNA pellet was resuspended in RNase-free water, and LiCl precipitated to remove residual unincorporated nucleotides. The pelleted RNA was resuspended in RNase-free water, and the quantity and purity were determined by UV spectrophotometry. An aliquot was electrophoresed on an agarose/formaldehyde gel to determine the size range of the products.

Agarose/Formaldehyde Gel Electrophoresis and RNA Blotting

RNA was ethanol precipitated, dried, resuspended in gel loading buffer containing formamide and formaldehyde, and loaded onto a 1% agarose/formaldehyde gel. After electrophoresis, the RNA was stained with ethidium bromide and then transferred to Hybond-N (Amersham Pharmacia Biotech, Piscataway NJ) by electroblotting. The membrane was prehybridized in Rapid-hyb buffer (Amersham Pharmacia Biotech) at 68°C for 30 min. A 537-bp fragment of murine β-actin served as a template for random-primed probe generation. The ^{32}P-labeled probe was added to the membrane, and hybridization was at 68°C for 2 h, followed by one wash in 2× SSC, 0.1% SDS at room temperature for 20 min and two washes in 0.2× SSC, 0.1% SDS at 68°C for 10 min each. The membrane was exposed to film with an intensifying screen at −80°C.

Transfection of Murine and Human DCs with RNA

RNA, in 200 μl Opti-MEM, and the lipid DMRIE (Vical, San Diego, CA), in 200 μl Opti-MEM, were mixed in polystyrene tubes at room temperature for 5–10 min. The amount of CEA- or GFP-specific RNA used was 1 μg, total tumor RNA was 10 μg, and amplified tumor RNA was 5 μg, per 10^6 DCs. The RNA:lipid ratio was 1:3. The complex was added to DCs (5×10^6 cells/ml) in Opti-MEM (Life Technologies) and incubated at 37°C for 20–30 min. DCs were washed and used as stimulators.

CTL Priming in Mice

Bone marrow precursor-derived DCs were transfected with the various RNA preparations, and naive, syngeneic mice were immunized i.v. with 5×10^5 precursor-derived DCs/mouse in 200 μl of PBS, two times at 14-day intervals. Splenocytes were harvested 12–14 days after the final immunization and depleted of RBCs with ammonium chloride Tris buffer. Splenocytes (10^7) were cultured with 5×10^5 stimulator B16/F10.9 cells pretreated with IFN-γ and irradiated at 7500 rads in 5 ml of Iscove's modified Dulbecco's medium with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M β-mercaptoethanol/well in a six-well tissue culture plate. Cells were cultured for 5 days at 37°C and 5% CO_2. Effectors were harvested on day 5 on Histopaque 1083 gradient prior to use in a CTL assay.

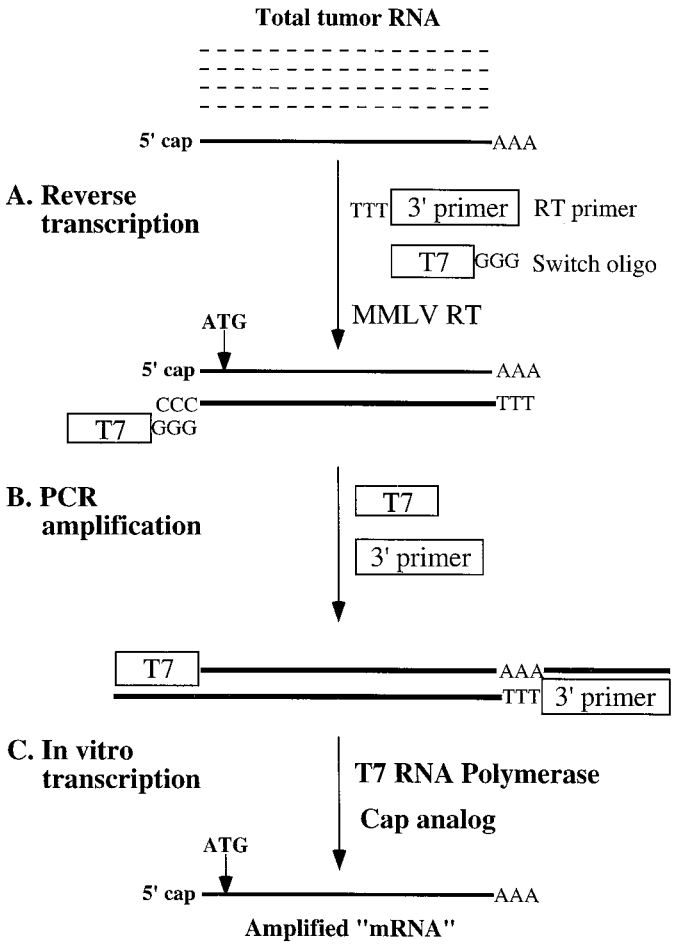

Fig. 1. RNA amplification. RNA from tumor cells was reverse transcribed using an RNase-minus Moloney murine leukemia virus (*MMLV*) RT that adds three to four C residues when it reaches the end of the RNA template. Reverse transcription was primed with an oligonucleotide containing 30 T residues (in later experiments, 64 residues) and a unique 23-nucleotide-long sequence needed for the subsequent amplification step (RT primer). A second 24-nucleotide-long oligonucleotide encoding the phage T7 promoter and 3 G residues (switch oligo) was used to extend the 3′ ends of the reverse transcripts. cDNA is amplified with a high fidelity DNA polymerase using as 3′ primer the unique sequence from the RT primer and as 5′ primer the T7 promoter oligonucleotide. This protocol was designed to enrich for full-length cDNA transcripts because only cDNA species that have extended to the 5′ end of their templates and incorporated the T7 sequences will be amplified. The amplified cDNA library was transcribed into RNA using T7 polymerase in the presence of a cap analogue, as described previously (12).

Induction of Antigen-specific Primary Human CTL Responses *in Vitro*

PBMCs were used as responder cells and resuspended in complete RPMI [RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M β-mercaptoethanol] at 2×10^6 cells/ml. Cells were cocultured with RNA-transfected DCs at a responder:stimulator ratio of 10:1 in 10–20 ml of complete RPMI and 10 ng/ml IL-7. IL-2 was added on day 3 at a concentration of 20 units/ml. Fresh medium was added every 5 days. Viable cells were harvested on day 12, and $CD8^+$ T cells were isolated using CD8 microbeads, per the manufacturer's protocol (Miltenyi Biotech, Sunnyvale, CA). The purity of $CD8^+$ T cells was routinely 90% by fluorescence-activated cell sorter analysis. The captured $CD8^+$ T cells were cultured in 10 ml of complete RPMI 1640 and 20 units/ml of IL-2 at 37°C. Two days later, the CD8+ T cell blasts were harvested and restimulated with RNA-transfected DCs. $CD8^+$ T cells were maintained at 5×10^5 cells/ml in complete RPMI, 10 ng/ml IL-7, and 20 units/ml IL-2. CTL assays were done 5–6 days after restimulation.

In Vitro Cytotoxicity Assay

Target cells ($5–10 \times 10^6$) were labeled with europium for 20 min at 4°C. Europium-labeled targets (10^4) and serial dilutions of effector cells at varying E:T were incubated in 200 μl of complete RPMI 1640. The plates were centrifuged at $500 \times g$ for 3 min and incubated at 37°C for 4 h. Fifty μl of the supernatant were harvested, and europium release was measured by time-resolved fluorescence (21). Specific cytotoxic activity was determined using the formula:

$$\% \text{ specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

Spontaneous release of the target cells was <25% of total release by detergent in all assays. Standard errors of the means of triplicate cultures was <5%.

RESULTS

Amplification of mRNA from Tumor Cells. To generate an amplified RNA product that can be efficiently translated in the DCs, the amplification protocol has to be highly efficient. The RT-PCR protocol used in this study is outlined in Fig. 1. A 3′-primer carrying a stretch of T residues was used to prime reverse transcription. Reverse transcription is the limiting step in generating full-length cDNAs and hence translatable RNAs. To enrich for cDNA products that extended beyond the initiation codon of the mRNA (Fig. 1, ATG), reverse transcription was carried out with the RNaseH-minus Moloney murine leukemia virus reverse transcriptase, which adds three to four C residues to the end of the cDNA strand when the enzyme reaches the 5′ end of the RNA. This property can be used to anchor an oligonucleotide, called "strand switch oligo," containing three G residues, and permit the extension of the cDNA (22, 23). The sequence of the strand switch oligo used in this protocol corresponds to the T7 phage promoter. The resulting cDNA is amplified using the 3′ primer (without the T residues) and the T7 promoter-containing oligo (without the G residues) as the 5′-primer in a standard PCR reaction. The amplified cDNA product is used as the template for RNA transcription using T7 RNA polymerase in the presence of a 5′-cap

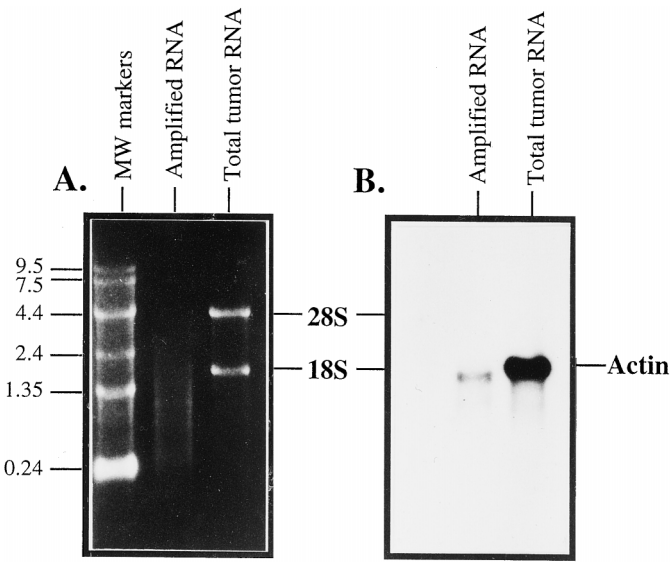

Fig. 2. Biochemical analysis of RNA amplified from B16/F10.9 tumor cells. Total RNA was isolated from B16/F10.9 cells using the RNeasy kit (Qiagen), following the manufacturer's protocol. One μg of RNA was amplified as described in Fig. 1 and "Materials and Methods." Tumor RNA and the amplified RNA products were subjected to agarose/formaldehyde gel electrophoresis and stained with ethidium bromide (*A*). Alternatively, RNA was blotted and hybridized with a murine actin probe (*B*).

analogue, as described previously (12). The procedure, described in details in "Materials and Methods," is completed in <24 h.

The ability to amplify tumor RNA was first tested in the B16/F10.9 melanoma tumor model. The B16/F10.9 melanoma tumor is poorly immunogenic, expresses low levels of MHC class I molecules, and is highly metastatic in both experimental and spontaneous metastasis models (18). RNA was isolated from B16/F10.9 tumor cells, and an aliquot was amplified as outlined in Fig. 1. RNA was analyzed before and after amplification by agarose gel electrophoresis. As expected, staining with ethidium bromide (Fig. 2*A*) shows that the predominant RNA species present before amplification correspond to the two rRNA species, whereas the amplified RNA migrates as a heterogeneous population corresponding in size to the mRNA population in murine cells. To assess whether the amplified RNA species correspond to full-length transcripts, the RNA displayed in Fig. 2*A* was blotted and hybridized with an actin-specific, random-primed probe. In each case, a single prominent band was seen that corresponds in size to the murine actin mRNA (Fig. 2*B*). Judging from the band intensities, we estimate that the efficiency of generating full-length actin during the amplification procedure was not >3–5%. Note also that the actin-specific RNA species in the amplified RNA preparation is slightly shorter than the actin RNA species seen prior to amplification. This would be expected because 54 nucleotides are added to the ends of the amplified RNA species (30 A residues + 23 residues corresponding to the 3′ primer) compared with ~200 A residues at the ends of cellular mRNA species.

DCs Transfected with Amplified RNA Prime CTL Responses *in Vivo* and Engender Protective Immunity in Tumor-bearing Mice. To determine whether the amplified RNA has retained immunological activity, RNA isolated from B16/F10.9 cells (preamplification RNA) and the amplified RNA (postamplification RNA) were used to prime a CTL response *in vivo*. Mice were immunized twice with RNA-transfected DCs; splenocytes were isolated, restimulated with IFNγ pretreated and irradiated B16/F10.9 cells, and tested for the presence of B16/F10.9-specific CTLs using a standard cytotoxicity assay (Fig. 3). As shown previously, irradiated B16/F10.9 were not capable of priming a CTL response, reflecting the poor immunogenicity of this tumor cell line (18). On the other hand, DCs transfected with tumor RNA isolated from B16/F10.9 tumor cells or the amplified RNA products stimulated a robust CTL response. The specificity of the CTL response is shown by the fact that B16/F10.9 but not EL4 tumor cells were lysed by the CTLs. The RT primer initially used in the amplification protocol described in the legend to Fig. 1 contained a stretch of 30 T residues to code for a 30-nucleotide long poly(A) tail in the amplified RNA. Experiments in our laboratory have shown that extending the poly(A) tail of *in vitro*-transcribed CEA RNA to 64 nucleotides enhances the ability of the CEA RNA-transfected DCs to stimulate a CTL response *in vitro*, presumably by increasing the stability of the RNA in the cell.[4] As shown in Fig. 3, CTL priming was improved by extending the poly(A) tail of the amplified RNA from 30 to 64 residues. This difference was seen reproducibly in three experiments using different RNA preparations. Subsequent amplification protocols described below were therefore modified to generate the longer poly(A) tail.

Because the RT primer contains a stretch of T residues, the amplification protocol outlined in Fig. 1 should result in ~30-fold enrichment of mRNA sequences in the amplified product, reflecting the abundance of rRNA in the starting material. Yet, as shown in Fig. 3, both pre- and postamplification RNA preparations induce a comparable CTL response that cannot be explained by the fact that twice as much preamplification RNA was used. The likely explanation for this discrepancy, as indicated in Fig. 2, is the low yield of full-length translatable RNA species in the amplified product.

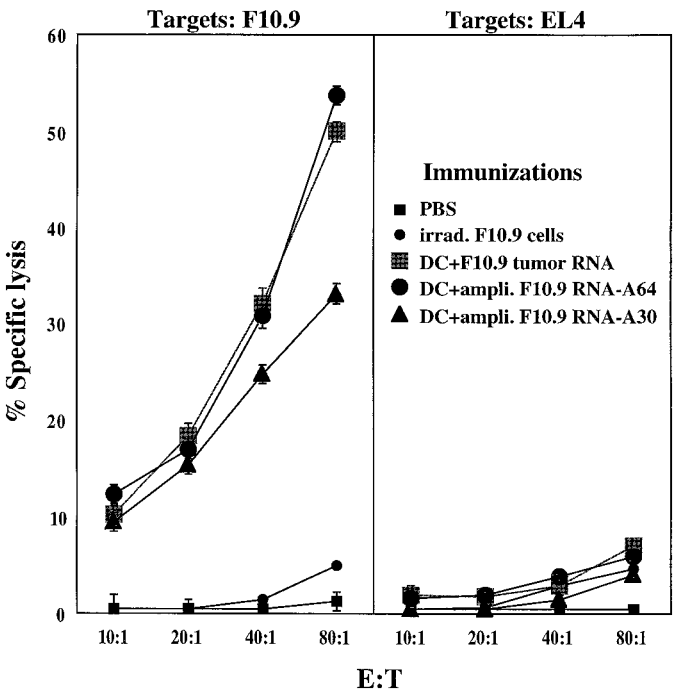

Fig. 3. Induction of a tumor-specific CTL response in mice immunized with DCs transfected with amplified tumor RNA. Mice were immunized twice with PBS, irradiated B16/F10.9 cells, or with DCs transfected with total tumor RNA or amplified RNA products. RNA was amplified with either of two RT primers containing 30 or 64 T residues. Splenocytes were harvested, restimulated with IFNγ-treated irradiated B16/F10.9 cells for 5 days, and tested for the presence of B16/F10.9-specific CTLs using a standard cytotoxicity assay. B16/F10.9 and EL4 cells were used as specific and nonspecific targets. The results are representative of four experiments. *Bars*, SD.

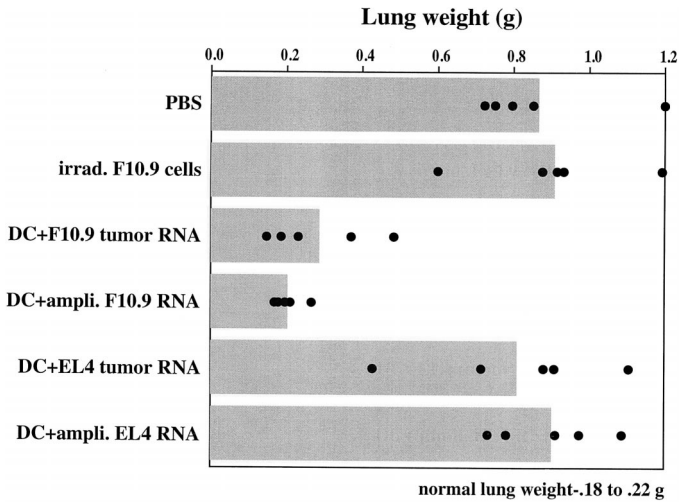

Fig. 4. Regression of lung metastases in mice treated with DCs transfected with RNA amplified from B16/F10.9 tumor cells. The postsurgical metastasis protocol was used as described previously with a few modifications. Mice were injected intrafootpad with 2×10^5 B16/F10.9 cells, and primary tumors were surgically removed under anesthesia when the local tumor in the footpad was 5.5–7.5 mm in diameter. Two days later, mice were immunized i.p., followed by two additional vaccinations at 14-day intervals, for a total of three vaccinations. Treatment groups included mice injected with PBS, or immunized with irradiated B16/F10.9 cells, DCs transfected with RNA isolated from B16/F10.9 and EL4 tumor cells, or with amplified RNA from the same sources using the RT primer containing 64 T residues. Mice were sacrificed based on the metastatic death in the PBS-treated group (25–30 days after removal of the primary tumors). Metastatic loads were determined by measuring lung weights. *Columns*, mean lung weight; *dots*, individual lung weight (five mice/group). These results are representative of four experiments.

[4] Unpublished data.

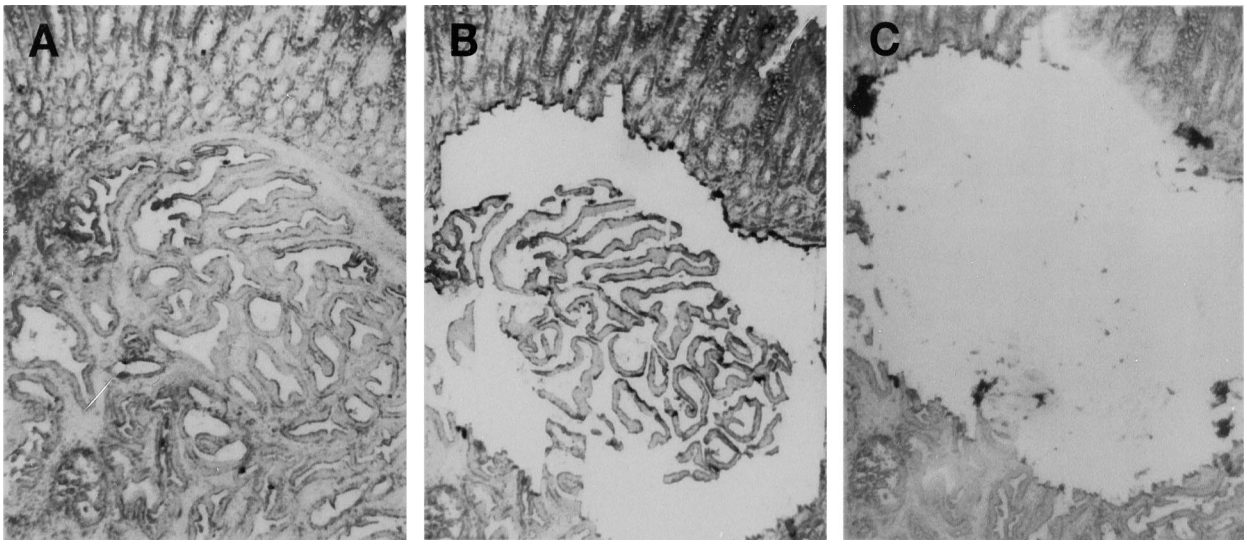

Fig. 5. Microdissection of a colorectal metastatic nodule from a frozen section. A liver metastasis was resected for curative intent from a patient with CEA-positive colorectal cancer. Frozen sections were cut at 20 nm in a cryostat mounted on glass slides and stained with H&E. A single tumor nodule can be seen in *A*, surrounded by normal tissue. Microdissection was performed using an inverted microscope and an attached mechanical micromanipulator for manipulating a glass capillary tube, which is used as a cutting tool. Under ×40-×400 magnification, tumor cells are separated from benign tissue by first removing the normal cells adjacent to the tumor nodule (*B*) and then collecting the tumor tissue in the middle (*C*). Thirty-five tumor nodules were isolated and pooled from two slides, and RNA was extracted using phenol/chloroform and ethanol precipitation. RNA yield, determined by UV absorption, was 2.24 μg. The integrity of the RNA was confirmed by agarose gel electrophoresis under denaturing conditions and ethidium bromide staining (data not shown).

To determine whether priming of a CTL response shown in Fig. 3 correlates with a protective antitumor immune response, we tested whether immunization of mice with amplified tumor RNA-transfected DCs was capable of causing the regression of preexisting lung metastases. Primary tumors were induced by implantation of B16/F10.9 tumor cells in the footpad of mice. When the tumor reached a certain size (5.5–7.5 mm diameter), they were surgically removed, and 2 days later, mice were immunized with irradiated B16/F10.9 cells or with various RNA-transfected DC preparations. Metastatic load was determined by measuring lung weight and visual inspection (the latter is less quantitative and more subjective). We have shown previously that in this model, treatment of the tumor-bearing mice with B16/F10.9 RNA-transfected DCs exerts a pronounced antimetastatic response (12). As shown in Fig. 4, the lung weight of mice injected with PBS or treated with irradiated B16/F10.9 cells quadruples to ~0.8 g (average normal lung weight is 0.2 g), reflecting the extensive metastasis occurring in this organ and the poor immunogenicity of B16/F10.9 tumor cells. On the other hand, immunization with DCs transfected with either RNA isolated directly from tumor cells or with amplified RNA exhibited a potent antimetastatic response. The specificity of this response is shown by the fact that DCs transfected with RNA from an unrelated tumor, EL4, had no effect. In the pre- and postamplification B16/F10.9 treatment groups, three and four animals, respectively, had normal lung weights and were free of visible metastases, whereas in the control groups, animals had multiple metastatic nodules that were too many to count. The therapeutic response seen in the group treated with amplified RNA was slightly superior to the group treated with nonamplified RNA. This difference was seen in three experiments using three different preparations of amplified RNA. The likely explanation, as discussed above, is that the amplified RNA was enriched for mRNA species 30-fold compared with the preamplification tumor RNA, which was offset in part by the limited efficiency of the amplification process.

DCs Transfected with mRNA Amplified from Colorectal Tumor Cells Obtained by Microdissection from Frozen Tissue Section Stimulate a Primary CTL Response *in Vitro*. The murine studies described in Figs. 3 and 4 show that RNA amplified from tumor cells retains biological function. In the cancer patient, the most readily available source of tumor is fixed tissue specimen. Examination of pathology slides can accurately distinguish tumor from benign tissue, and using microdissection techniques, tumor cells can be isolated with a high degree of purity. In the following experiments, we

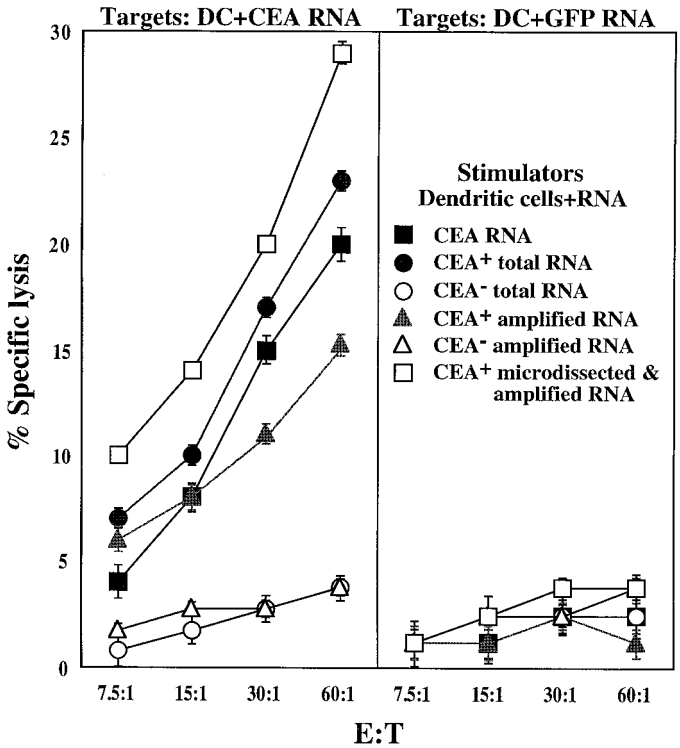

Fig. 6. Induction of a primary, CEA-specific CTL response *in vitro* using DCs transfected with RNA amplified from microdissected colorectal tumor cells. PBMCs from a healthy volunteer were stimulated with autologous DCs transfected with various RNA preparations and tested for the presence of CEA-specific CTL. DCs transfected with *in vitro* synthesized CEA RNA or GFP RNA were used as specific and nonspecific targets, respectively, as described previously (16). For stimulations, DCs were transfected with *in vitro* transcribed CEA RNA, RNA isolated from a CEA^+ cell line (SW1463), a CEA^- cell line (KLEB), RNA amplified from SW1463 and KLEB cells, and RNA amplified from the microdissected tumor cells described in Fig. 5. These results are representative of two experiments; *bars*, SD.

tested whether RNA isolated from tumor tissue microdissected from pathology slides can be amplified without loss of function. Fig. 5*A* shows an H&E-stained frozen section from a CEA^+ colorectal liver metastasis showing a metastatic nodule surrounded by benign tissue. To isolate tumor cells, normal tissue surrounding the cancerous nodule is first removed (Fig. 5*B*), and then the tumor cells are collected (Fig. 5*C*). Thirty-five nodules were isolated in a 3-h procedure from two consecutive sections and pooled, and RNA was extracted. RNA yield was 2.24 μg, obtained from ~55,000 tumor cells. Gel analysis under denaturing conditions and ethidium bromide staining confirmed that the RNA was intact (data not shown).

We have shown previously that human monocyte-derived DCs transfected with CEA-specific RNA transcribed *in vitro* from a cDNA plasmid, or with total RNA isolated from CEA-expressing cell lines, were capable of stimulating a CEA-specific CTL response *in vitro* (16). Because the RNA amplified from the microdissected tissue was derived from a CEA^+ colorectal tumor, we measured the induction of CEA-specific CTL response to determine whether the amplified RNA was immunologically active. DCs were generated from a healthy volunteer, transfected with RNA, and incubated with autologous PBMCs. After two rounds of stimulation, the presence of CEA-specific CTLs was determined using autologous DCs transfected with CEA RNA or with GFP RNA as specific and nonspecific targets, respectively. As shown in Fig. 6, DCs transfected with *in vitro* synthesized CEA RNA or with RNA isolated from a CEA-positive cell line, but not from a CEA-negative cell line, was capable of stimulating a CEA-specific CTL response. An aliquot of the RNA isolated from the CEA-positive and the CEA-negative cell lines was amplified as outlined in Fig. 1. As shown in Fig. 6, DCs transfected with amplified RNA from the CEA-positive, but not from the CEA-negative, cell line stimulated a CEA-specific CTL response. The reduced magnitude of the CTL response generated with the amplified RNA may represent either inefficient amplification or low level of CEA expression in the cell line. Strikingly, a robust CTL response was also generated using DCs transfected with RNA amplified from the microdissected tumor cells. This experiment clearly shows that tumor RNA can be amplified from tumor cells microdissected from tissue sections without loss of immunological activity. The significance of the differences seen in the magnitude of the CTL response stimulated with the various RNA preparations is not clear and is under investigation.

DISCUSSION

Unique patient-specific tumor antigens that have not triggered tolerance may constitute the dominant antigens in the antitumor immune response (6). Hence, vaccination with the patient's own repertoire of tumor antigens may offer a superior strategy to elicit protective immunity. This approach is currently limited to a select group of patients from whom sufficient tumor tissue can be obtained for antigen preparation. We have shown previously that DCs transfected with tumor RNA stimulate potent class I-restricted CTL responses and engender protective immunity in tumor-bearing mice (12, 13, 16). In the current study, we have shown that tumor RNA isolated from murine tumor cell lines and from primary human tumor cells obtained by microdissection can be amplified without loss of function. The potency of vaccination with tumor RNA-transfected DCs as indicated in our preclinical studies, combined with a method to generate adequate amounts of antigen from microscopic amounts of tumor tissue, provides the foundation for an effective and broadly applicable treatment that is not encumbered by the need to know the antigenic profile of each cancer patient or the availability of sufficient tumor tissue for antigen preparation.

The amplification protocol outlined in Fig. 1 is simple and rapid; the procedure involves well-characterized biochemical reactions carried out in a test tube and is completed in <24 h. In the protocol used in this study, RNA is amplified from 1 μg of total cellular RNA, which corresponds to ~40,000 cells. We have also successfully amplified RNA from as little as 50 ng of tumor RNA (corresponding to ~2000 cells). A useful feature of this protocol is that the 5′ primer used in the amplification step encodes the phage T7 promoter. As a result, the amplified cDNA library serves as the direct template for the *in vitro* transcription of the RNA product. DCs transfected with amplified cDNA instead of RNA would require the cloning of the cDNA library into an expression plasmid (to provide a eukaryotic promoter), which would be prohibitively complex to perform for each patient.

Gel analysis and ethidium bromide staining reveals that the size distribution of the amplified RNA products corresponds to that of the mRNA population in the cell (Fig. 2*A*). However, when a specific RNA species was analyzed by blotting and hybridization, only a fraction of the transcripts appear to be full length and hence translatable (Fig. 2*B* and data not shown). Yet, the amplified RNA product appears to be equally or more effective than the preamplified cellular RNA in stimulating CTL and protective antitumor immunity (Figs. 3, 4, and 6). The likely explanation is that the amplification protocol results in about 30-fold enrichment of the relevant [$poly(A)^+$] RNA population, which compensates in part for the low frequency of the translatable RNA species. Although current studies focus on improving the amplification protocol, from a vaccination standpoint, the limited efficiency of the amplification protocol is not a significant hindrance because generating sufficient amounts of RNA is not a limiting factor.

The ability to amplify RNA from microdissected tissue illustrates the power of the technology to provide an unlimited supply of antigen for vaccination from a limited source of tumor tissue. Tumor cells are often intertwined with benign tissue at the microscopic levels, as illustrated in Fig. 5*A*. Using microdissection techniques, tumor cells can be isolated with a high degree of purity, preventing the dilution of tumor antigens and reducing the potential of inducing autoimmunity against self antigens from surrounding tissue. Concern that vaccination with undefined antigenic mixtures from tumor cells may also break tolerance to self antigens expressed in the tumor cells may be addressed in the future by using subtractive hybridization techniques (24, 25), illustrating yet another potential advantage of using tumor mRNA as antigen.

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Induction of Tumor Immunity and Cytotoxic T Lymphocyte Responses Using Dendritic Cells Transfected with Messenger RNA Amplified from Tumor Cells

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