T-Cell Receptor Transgenic Analysis of Tumor-specific CD8 and CD4 Responses in the Eradication of Solid Tumors

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ABSTRACT

The role of tumor-specific CD8 and CD4 lymphocytes in rejecting solid tumors has been difficult to determine because of the lack of models in which tumor antigen, specific CD8 cells, and specific CD4 cells can be monitored and controlled. To investigate the minimal components required for the induction and maintenance of CTL activity sufficient to reject a solid tumor in vivo, we transfected the influenza hemagglutinin (HA) gene into a nonimmunogenic class I/class II murine malignant mesothelioma (MM) tumor line to generate an endogenous tumor antigen and used TCR transgenic mice with class I- or class II-restricted specificities for HA as sources of naive, tumor-specific T cells. The data show that the presence of a strong tumor antigen is not in itself sufficient to induce an effective CTL response, nor does the presence of a high frequency of precursor cells guarantee tumor rejection. We also show that tumor-specific CD4 cells, when CTL numbers are suboptimal, greatly enhance the eradication of tumor, confirming the importance of antigen-presenting cell presentation of tumor antigens to class II-restricted cells. These data confirm that T-cell receptor transgenic cells, combined with nominal tumor antigen transfection, represent powerful tools to analyze tumor-specific T-cell responses.

INTRODUCTION

Various studies have examined the mechanisms by which tumors evade the immune response. These mechanisms include lack of tumor antigen expression, down-regulation of surface MHC molecules or loss of peptide processing components, production of suppressor factors, physical exclusion from the tumor site, and lack of costimulatory molecules (3, 4). Thus, despite these obstacles, the immune system can respond to and, in some cases, eradicate tumors in animals and in humans (11, 12). Nevertheless, these examples appear to be the exception rather than the rule. Before we can successfully harness the immune system in cancer treatment and prevention, we need to understand why antitumor responses are usually so ineffective.

In general, the focus of studies investigating or manipulating the immune response against tumors has been the generation of CTLs because of the lytic nature of these cells. Yet tumor-specific CTLs can be demonstrated in the absence of in vivo effects (13). Such contradictory evidence may simply reflect a difference between in vitro and in vivo measurements but also implies that the generation of CTLs is not the sole functional effector required for antitumor immunity. In fact, an increasing body of evidence suggests that CD4 cells can play a critical role in the eradication of tumors (14). CD4 cells have been implicated indirectly in experiments that replace “help” by introducing IL-2 (15). It has also been shown that tumors transduced with syngeneic class II molecules can induce protection against parental class II-negative tumors (16).

Many of these studies have been carried out in the absence of a known tumor antigen and have therefore lacked the capacity to follow the fate of tumor-specific T cells. Even when tumor antigens are defined, it has been difficult to understand the relationship between tumors and host tumor-specific CD8 and CD4 cells because of the lack of a model in which all of these parameters can be defined, controlled, and monitored. To investigate the requirements for effective immune recognition of a solid tumor, we have generated a defined model system using a transfected antigen (influenza HA) and TCR transgenic mice. HA is an ideal nominal tumor antigen because its class I and class II peptide reactivities are well defined and, importantly, anti-HA TCR transgenic mice are available with both class I and II specificities. Two lines of TCR transgenic mice, one class I restricted (CL4 mice; Ref. (17)), the other class II restricted (HNT mice; Ref. (18)), both of which recognize HA in the context of H-2d, are used in this study. The advantage of using these TCR transgenic mice is that they provide a virtually monoclonal source of cells of known specificity, where the respective roles of tumor-specific CD8 and CD4 cells can be evaluated. Thus, either in intact animals or through adoptive transfer experiments, it is possible to investigate how the frequency of tumor-specific cells influences tumor immunity and the role that specific CD4 cells play in modulating the response in the absence of direct tumor recognition. Using this new model, we show that tumor-specific CD4 cells act synergistically with limiting antitumor CTLs to prevent the growth of a solid class II+ tumor.

MATERIALS AND METHODS

Mice. BALB/c (H-2d) and BALB/c nu/nu mice (SPF, female, 6–8 weeks of age) were obtained from the Animal Resources Center (Western Australia, Australia) and maintained under standard conditions in the University Department of Medicine animal housing area. Two lines of anti-HA transgenic TCR mice were used. The HNT line is class II restricted and recognizes its epitope PR/8 HA; 126–138 in the context of I-Ab. The CL4 line is restricted by H-2Kd and recognizes residues PR/8 HA; 533–541. The derivation of the CTL clone-4 TCR transgenic line (CL4) has been described previously, and breeding pairs were obtained from Drs. L. Sherman and D. Lo (The Scripps Research Institute, La Jolla, CA; Ref. (17)). The HNT line has also been described previously (18). Both transgenic lines were backcrossed for five generations onto the BALB/c genetic background and bred in the University SPF animal facility. Experimental animals were maintained in a non-SFF holding facility.

Murine MM Tumor Cell Line. The derivation and characterization of the AB1 murine MM cell line used in this study have been described previously (19). Essentially, the cells were generated by inoculating crocidolite asbestos i.p. into H-2d (BALB/c) mice, and peritoneal exudate was passaged in vitro and in vivo until stable clonal cell lines were obtained. Cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin (CSL, Melbourne, Victoria, Australia), 50 μg/ml gentamicin (David Bull Labs, Victoria, Australia), and 5% FCS (Life Technologies, Inc.). AB1 is class I+, class II- and poorly immunogenic (20).
HA Transfectant Cells. The HA gene from the Mt. Sinai strain of the PR8 influenza virus was subcloned into the β-actin expression vector (pHB-Apr-neo; obtained from Dr. J. Allison, University of California, Berkeley, CA). AB1 (1 x 10^5) cells were seeded into six-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) and cultured until 60% confluent. For each well, 5 μg of plasmid DNA and 30 μl of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-methylsulfate transfection reagent (Boehringer Mannheim, Mannheim, Germany) were separately diluted to 250 μl in HEPES-buffered saline (140 mm NaCl, 2 mm KCl, 10 mm Na2HPO4, 2 mm KH2PO4, and 2 mm HEPES, pH 7.4) in sterile polystyrene tubes (Becton Dickinson). Both solutions were mixed together, incubated at room temperature for 10 min, and made up to 1 ml with Optimem (Life Technologies, Inc.). Spent media was aspirated from the cell cultures, and the transfection solution was added for 24 h. Transfected cells were selected by culturing in media containing the neomycin analogue geneticin (Life Technologies, Inc.) at a final concentration of 400 μg/ml. Cells were trypsinized, resuspended in 1 ml PBS supplemented with 5% FCS, and counted using trypan blue exclusion as a measure of cell viability. A 1-ml aliquot of cell suspension was serially diluted in media and seeded into 96-well round-bottomed plates at a concentration of 0.3 cells/well in a total volume of 200 μl/well. Cells were cultured for 7–10 days and examined daily for cell colony formation. Wells containing a single colony were trypsinized and subcloned into separate wells of a 24-well plate. When ~70% confluent, cultures were trypsinized and seeded into 80-cm² tissue culture flasks (Becton Dickinson, Lincoln Park, NJ) and routinely maintained in media containing 400 μg/ml G418. The level of HA expression on transfected cells was measured by FACS analysis, using the biotinylated HA-specific monoclonal antibody H18 (21) and was originally obtained from Dr. Walter Gerhard (The Wistar Institute, Philadelphia, PA). HA transfectant clones were enriched by Dynal beading. Cells were incubated (4°C for 30 min) in 1:500 dilution of anti-HA antibody and then washed three times in media (RPMI+5% FCS) and incubated with streptavidin beads (Dynal; 4°C for 30 min). Cells were washed three times, and beaded cells were separated from the cell mix using a magnet.

Characterization of the in vivo Growth of the Transfected Tumor. Groups of 20 female BALB/c or BALB/c nu/nu mice, 6–8 weeks of age, were inoculated s.c. with either 1 x 10^6 AB1-HA transfectants with relatively high HA expression (AB1-HA LO) or those with relatively low expression (AB1-HA HI) and monitored for tumor growth for up to 2 months after inoculation. HNT and CL4 TCR transgenic mice were inoculated s.c. with the same lines or with parental control lines and also monitored for tumor growth for up to 2 months after inoculation. Tumors between 0.5- and 1.0-cm diameter were excised and tested for maintenance of HA expression using FACS analysis.

Winn Assay. To evaluate antitumor activity in vivo, we used a Winn assay (22). Cells from the draining LNs or contralateral LNs (4 x 10^6) of class 1-restricted CL4 TCR transgenic mice that had developed AB1-HA tumor were mixed with either AB1-HA LO cells or the parental AB1 tumor (1 x 10^7) and injected s.c. into BALB/c recipient mice, and the animals were monitored for tumor development. In Vitro Generation of HA-specific CTLs. Effector cells were generated by incubating spleen cells from anti-HA CL4 TCR transgenic mice with peptide (1 μg/ml) for 5 days. Target cells included MM cell lines transfected with HA, P815, a chemically induced mastocytoma in DBA/2 mice obtained from the American Type Culture Collection (Rockville, MD) and untransfected MM cell lines. Targets were labeled with 150 μCi of 51Cr for 90 min and washed four times before use. Effector cells were added to corresponding targets at varying E:T ratios and incubated at 37°C for 4 h. After incubation, the supernatants were harvested, and radioactivity was counted in a gamma counter. The mean of triplicate samples was calculated, and the percentage of specific 51Cr release was determined as follows:

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\text{Percentage of specific cytotoxicity} = \frac{\text{Experimental} \ 51\text{Cr} \text{ release} - \text{control} \ 51\text{Cr} \text{ release}}{\text{Maximum} \ 51\text{Cr} \text{ release} - \text{control} \ 51\text{Cr} \text{ release}} \times 100
\]

Experimental 51Cr release represents counts from target cells mixed with effector cells, control 51Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum 51Cr release represents counts from targets exposed to 5% Triton X-100.

Incubation of Tumor Cells with IFN-γ. Tumor cell lines were treated as monolayer cultures for 48 h with 100 units/ml of recombinant mouse IFN-γ (Genzyme, Cambridge, MA) with a specific activity of 1.14 x 10^6 units/μg protein and washed three times in media before use in in vitro assays.

Preparation of HA-specific, Class I-restricted (CL4), and Class II-restricted (HNT) Cells for Adoptive Transfer. LN cells were purified first over nylon wool and then depleted either of CD4 T cells (CL4 mice) or CD8 T cells (HNT and BALB/c mice). BALB/c mice were then injected with 1 x 10^7 cells i.v. Twenty-four h after adoptive transfer, mice were inoculated s.c. with 2 x 10^5 AB1-HA tumor cells.

Depletion of CD8 and CD4 T Cells. For in vitro depletion of either CD8 T cells or CD4 T cells, nylon wool-purified T cells were exposed to a 1:3 dilution of 3.168 (anti-CD8) or RL172 (anti-CD4) supernatant in RPMI with 5% FCS for 30 min at 4°C, washed once, and then exposed to a 1:10 mixture of rabbit complement (C-six Diagnostics, Inc., Mequon, WI) in RPMI with 10% FCS for 30 min at 37°C. Cells were then washed twice, and purity was checked by FACS analysis using FITC and phycoerythrin-conjugated anti-CD8 or anti-CD4 (PharMingen, San Diego, CA). Analysis was performed on a FACSscan (Becton Dickinson) using Cell Quest software.

5- and 6-) CSFE Labeling of CL4 TCR Transgenic LN Cells. CSFE labeling was performed as described previously (23). Tumor-specific (HA-specific) LN cells, from the TCR transgenic class I-restricted CL4 mice, were resuspended in PBS at 1 x 10^7 cells/ml. For labeling, 2 μl of a CSFE (Molecular Probes, Eugene, Oregon) stock solution (5 mM in DMSO) were incubated with 10 ml of cells (at 10^7/ml) in RPMI 1640, without FCS, for 10 min at room temperature. Cells were washed through FCS four times and then resuspended in PBS before injecting 1 x 10^7 cells into recipients i.v. In all experiments, cells were recovered 3 days after transfer and analyzed by FACS analysis.

Proliferation Assay. BALB/c mice were inoculated s.c. with the AB1-HA LO cells, and LN cells from CL4 and HNT mice (1 x 10^7 cells each) were adoptively transferred i.v. Draining and non-draining LNs from the recipient mice were isolated 2 and 8 weeks later. Single cells suspensions were made, and each sample was divided into two, one of which was depleted of CD8 cells, and the other was depleted of CD4 cells. B cells were depleted from all samples. Cell depletions were done using the anti-CD8 or -CD4 antibodies and the B220 antibody (PharMingen), followed by magnetic bead separation using beads coated with sheep anti-rat immunoglobulin (Dynal, Oslo, Norway). Cells (1 x 10^6) were plated in triplicate in 96-well, flat-bottomed microtiter plates (Costar, Cambridge, MA) in a final volume of 200 μl of RPMI/10% FCS, plus or minus the appropriate peptide (CL4 peptide in CD4-depleted samples; HNT peptide in CD8-depleted samples). Plates were incubated for a total of 48 h, and 3H was added 12–15 h before harvesting.

RESULTS

The Expression of a Strong Antigen Is Not Sufficient to Initiate an Effective Immune Response to a Solid Tumor. The MM cell line AB1 transfected with plasmid DNA encoding the influenza virus HA molecule under the control of a β-actin promoter was cloned by limiting dilution and selected for variants with different levels of surface HA expression (LO and HI; Fig. 1, A and B). To confirm that these processes did not alter the tumorigenicity of the transfectants, immunodeficient BALB/c nude mice were inoculated s.c. with the transfectants and the parental line. All tumor lines grew with similar kinetics (Fig. 1E). When either transfectant was inoculated into wild-type BALB/c mice, tumors formed in all experimental groups, although the rate of engraftment was delayed in animals that received the AB1-HA HI line (Fig. 1F). All mice inoculated with the AB1-HA LO line developed tumor by day 22, whereas all mice that developed tumor when inoculated with AB1-HA HI did so by day 38 (19 of 20 mice). We were unable to detect antitumor CTL responses in BALB/c mice to either the parental or the transfectant lines (data not shown). Histological analyses of the tumors showed very few infiltrating lymphocytes in any of the animals (data not shown). When tumor biopsies were disaggregated, cultured overnight, and analyzed for expression of HA by FACS, neither the AB1-HA HI nor AB1-HA LO lines expressed significant levels of HA on the tumor biopsies.
tumor cell line had changed their pattern of expression during growth in vivo (Fig. 1, C and D). In addition, in vivo tumors, when subsequently analyzed by flow cytometry, did not demonstrate any alteration in class I expression, nor was the expression of class II molecules induced (data not shown).

A High Precursor Frequency of Potentially Tumor-reactive Cells Does Not Guarantee Complete Tumor Destruction. Given the apparent lack of immune recognition of the HA-transfected tumors in normal mice, we investigated growth of the transfectants in two situations where the frequency of class I-restricted, tumor-specific effectors is higher than that of naive BALB/c mice. HA-specific TCR transgenic mice have the advantage of containing a large number of cells of the same specificity, provide a large pool of “tumor-specific T cells” i.e., cells that recognize the antigen transduced into, and expressed exclusively in, the tumor cell. T cells (1 x 10^7) from an HA-specific, class I-restricted TCR transgenic line (CL4 mice) were adoptively transferred into BALB/c recipients, which were inoculated s.c. 24 h later with the AB1-HA^{LO} tumor. By day 20, 90% of the control animals, which received syngeneic T cells, had developed tumor (Fig. 2A). All control animals developed tumor by day 40. In contrast, mice that received CL4 T cells showed moderate but significant protection because ~40% of the experimental group rejected the tumor (Fig. 2A). To further increase the frequency of antitumor effectors, the AB1-HA transfectants were injected s.c. into the CL4 transgenic mice (Fig. 2B). Surprisingly, despite the fact that the vast majority of the CD8 cells in these animals are antigen specific, overwhelmingly more than would occur physiologically, there was little enhancement in the level of protection (50–70%; Fig. 2B) compared with the animals that received the adoptively transferred cells. Tumors that developed in the remaining CL4 TCR transgenic mice did so by days 30–36. Unlike BALB/c mice, both transfectants engrafted with similar kinetics in the CL4 transgenic mice, regardless of the level of HA expression on the tumor. Neither HA nor class I expression was lost when the tumor was excised and analyzed (data not shown).

Tumor Growth in the Class I-restricted CL4 TCR Transgenic Mice Is Not Due to Tolerization of the T Cells. LN cells from the CL4 mice in which the AB1-HA^{LO} tumor grew were mixed with transfectant cells and inoculated s.c. into naive BALB/c recipients (the Winn assay). Under these conditions, mice remained tumor free until the termination of the experiment at day 72, indicating that no tolerization of these CD8 effectors had occurred (Fig. 3A). Similar results were obtained when nondraining LN cells were used (data not shown). Control animals, which were inoculated with AB1-HA^{LO} (Fig. 3A) or parental tumor (Fig. 3B) only, developed tumor by day 30. Animals that received a mixture of the CL4 cells and AB1 cells developed tumor at the same rate as the control animals, indicating that the eradication of the AB1-HA tumor by the CL4 cells was specific (Fig. 3B).

The in Vivo Efficacy of the Class I-restricted CL4 Effectors Is Not Mirrored by in Vitro Assays of CTL Activity. Although MM lines constitutively express detectable levels of MHC class I molecules, exhibiting a mean fluorescence intensity as analyzed by FACs analysis of between 62 and 85 (Fig. 4A), it was possible that the inability to demonstrate HA-specific CTLs in AB1-HA-bearing BALB/c mice was due to lack of recognition of tumor peptide/class I complexes. We therefore investigated whether the transfected cells could be killed by CTLs isolated from the class I-restricted TCR transgenic mice. The CTLs were fully functional in that they could kill conventional peptide-pulsed targets, the mastocytoma, P815. (Fig. 5, A–C) and could also kill peptide-pulsed parental AB1 cells (Fig. 5C) and peptide-pulsed daughter HA transfectants (Fig. 5, A and B), indicating the presence of functional class I on these tumor lines. Surprisingly, the HA-transfected targets were not killed in the absence of added exogenous peptide (Fig. 5, A and B). Because this implied that the concentration of processed antigen delivered to class I molecules in the HA transfectants was insufficient for recognition by CTLs, we tested the effect of IFN-γ-induced class I up-regulation on the outcome of this assay. Incubation of the MM cells with IFN-γ induced an ~3-fold increase in the level of surface class I expression (mean fluorescence intensity, 219–286; Fig. 4B); in contrast, AB1-HA cells incubated with CL4 peptide did not increase the level of class I surface expression (data not shown). The up-regulation of class I expression did not significantly alter the level of killing of peptide-pulsed targets, either P815 or the transfected MM cells (Fig. 5, D–F); the killing of the AB1-HA cells was dramatically increased (Fig. 5, D and E).

A High Precursor Frequency of Tumor-specific CD4 T Cells Provides Little Protection against Tumor Growth. Although the MM cell lines are class II negative and therefore unable to directly interact with CD4 cells through the TCR, CD4 cells have been reported to have a number of nonspecific antitumor effects (reviewed in Ref. (14)). We tested the ability of tumor-specific CD4 cells to reject the AB1-HA tumors in an analogous manner to the experiments with the CL4-derived effectors. HNT cells, adoptively transferred into naive BALB/c recipients, demonstrated a slight ability to protect 30% of the recipients from subsequent tumor growth (Fig. 6A). Interestingly, protection against the AB1-HA cells was even less in the HNT transgenic mice, with all mice receiving AB1-HA^{LO} cells rapidly
developing tumor by day 12 and 80–90% of the AB1-HAHI inoculated animals developing tumor by day 30 (Fig. 6B). One explanation for these apparently conflicting data may be that the transgenic CD4 cells are “helping” an endogenous CD8 repertoire to reject the tumor. In the TCR transgenic mice, the endogenous CD8 repertoire is, perforce, restricted; therefore, the outcome is reduced in comparison to the adoptively transferred experimental group.

Enhanced Tumor Killing Can Be Achieved in Vivo by the Provision of Tumor Antigen-specific CD4 Cells. Although the transfected tumor lines were not lysed in vitro by specific CTLs, tumor was rejected in 60% of CL4 animals (Fig. 2B). These data, and those described above, suggest that mechanisms are in place in vivo that can drive the system to a threshold that allows CTL tumor recognition and destruction. Because a high precursor frequency appeared to be insufficient, even in some of the class I-restricted TCR transgenic mice, we tested whether CD4 cells could potentiate the antitumor response when CD8 cells were limiting. BALB/c mice were inoculated i.v. with CL4 plus HNT LN cells. One day later, the AB1-HALO tumor was inoculated s.c. and tumor growth was compared with that in animals that received tumor only (Fig. 7A). The results show that mice that received both the class I- and class II-restricted HA specific T cells were completely protected from tumor growth. This experiment was repeated using purified CD4 and CD8 populations, and similar results were obtained (data not shown).

When syngeneic CD4 cells were substituted for HNT cells, the level of protection was the same (40%; Fig. 7A) as in animals that received CL4 cells only (Fig. 2A). This implies that tumor-specific CD4 cells are required for complete tumor eradication.

Tumor-specific CD4 T Cells Proliferate in LNs Draining the AB1-HA Tumor. Because MM is a class II negative tumor yet tumor-specific CD4 cells improve tumor eradication in the adoptive transfer experiments described above, it was important to determine whether tumor-specific CD4 T cells recognize and proliferate to tumor antigen in vivo. Therefore, LNs from HNT TCR transgenic mice were isolated; the cells were labeled with the fluorescent dye CFSE and injected i.v. into AB1-HA or AB1 inoculated BALB/c mice (1 x 10^6 cells/s.c.). Three days after transfer of HNT cells into the various recipients, lymphoid tissues were removed and prepared for FACS analysis. CFSE labeling allows the detection of cellular proliferation by flow cytometry, with each daughter cell retaining one-half the original fluorescence of the parental cell. The fluorescence profiles of the CD4 cells from the draining LNs and contralateral LNs were compared (Fig. 7, A and B). Proliferation was only observed in the draining LNs (57% of CFSE labeled cells are in the proliferating peaks; Fig. 7A). No proliferation was seen in the nondraining LN (Fig. 7B) nor in the draining and nondraining LNs of animals inoculated with the parental AB1 tumor (data not shown). Importantly, this proliferation of tumor-specific CD4 cells in the tumor-draining LNs occurred in the absence of detectable tumor as determined by histology, PCR analysis of the transfected gene, or outgrowth of tumor cells from LNs cultured ex vivo (data not shown).

Tumor-specific T Cells Are Functionally Responsive ex Vivo. CD8 and CD4 cells were purified from the LNs of tumor-bearing animals 2 and 8 weeks after the coadoptive transfer of CL4 and HNT...
cells into tumor-inoculated recipients. The purified cells were tested for their ability to proliferate to the class I- or class II-restricted HA peptides. Cells responding to both peptides were detected, although the number of responsive cells decreased with time (Fig. 7C). When nontransgenic T cells were coadoptively transferred in this system and tested for their ability to proliferate to the class I- or class II-restricted HA peptides, the response was comparable with background levels, 667 ± 48 cpm and 701 ± 182 cpm, respectively.

**CD4 “Help” Does Not Potentiate CD8 Effector Cell Proliferation.** To determine whether the tumor-specific CD4 cells augmented the antitumor effects of the CD8 cells by increasing their initial in vivo expansion, CFSE-labeled CL4 cells were adoptively transfected, with or without concomitant transfer of HNT cells, into BALB/c mice that had been inoculated 14 days previously with AB1-HA<sup>HI</sup>. Three days after the adoptive transfer, the CFSE fluorescence profiles of CL4 cells, reisolated from animals in the different experimental groups, were analyzed (Fig. 8). CL4 cells interact with and proliferate to tumor antigen only in the LN draining the site of tumor inoculation<sup>4</sup> (Fig. 8A). Nondraining LNs are not a site of tumor antigen presentation to class I-restricted T cells (Fig. 8B). The coadministration of HNT cells did not cause any change in the “burst” size of the CD8 cells entering the proliferative phase after antigen recognition (Fig. 8C) because the profile of fluorescence was almost identical to that seen in Fig. 8A. Identical results were observed in BALB/c mice inoculated with AB1-HA tumor 21 or 28 days before transfer of the T cells (data not shown).

**Fas Expression Is Not Altered on CD8 Cells in the Presence of CD4 Cells and Tumor Destruction.** Activated cells can proliferate, express Fas (CD95) on the cell surface, and die. This sequence of events helps to limit the response to antigen but, in the case of tumor destruction, may check a useful response before it has eradicated the tumor. Although the expression of Fas, and the subsequent ligation of this molecule, leads to the deletion of activated cells, CD4 cells have been shown to inhibit their deletion (24). To test whether these two processes were linked in our model, the level of surface Fas expression on tumor-specific CD8 cells was determined in the presence or absence of tumor-specific CD4 cells using the adoptive transfer protocol described above with CFSE-labeled CL4 cells. Fas was chosen as a surrogate marker of cells programmed to die. Cells were analyzed 3 days after transfer into animals inoculated 14 days previously with AB1-HA<sup>HI</sup> tumor. When tumor-specific CD8 cells were transferred alone, Fas was expressed on 30% of nondividing cells (Fig. 9, peak D0), which increased to >90% of the cells in the last identifiable proliferative peak (D6). Almost identical results were obtained when HNT cells were cotransferred with the CL4 cells (Fig. 9) or when the experiment was conducted at earlier time points after tumor inoculation (days 7 and 10; data not shown).

**DISCUSSION**

**Expression of a Strong Tumor Antigen by Itself Is Not Enough to Induce Rejection.** There is an increasing endeavor directed toward the identification of tumor antigens, predominantly in the hope that it will be possible to use such antigens to augment antitumor responses. Although some form of response is initiated to tumors, as exemplified by the identification of tumor antigens, through the isolation of T cell clones derived from tumor-bearing mice (25), such examples appear to be the exception rather than the rule. In our experiments, the failure of BALB/c to reject the HA transfectants, despite a relatively high level of antigen expression, demonstrates that the normal complement of anti-HA precursor T cells is insufficient to generate a successful antitumor response. These results are similar to those from several other studies in which the model tumor antigens LCMV, SV40T, ovalbumin, or β-galactosidase have been evaluated (26–29). In most of these systems, the tumor was not rejected in unmanipulated animals, despite the continued expression of the antigen in the tumor cells. Thus, the HA molecule behaves like a typical human tumor antigen, present but not sufficient in itself to induce rejection, and thus provides us with a tool to start to examine the various roles of class I- and class II-restricted specific T cells in antitumor immunity.

**Tumor Eradication Is Not Dependent Simply upon CD8 Effector Frequency.** The immune system may not be able to eliminate tumor cells because the rate of tumor proliferation outstrips the capacity of the immune system to respond effectively before the tumor becomes established. Thus, the inability of BALB/c mice to reject the AB1-HA tumors may simply be due to a limited frequency of CD8 effector cells. This hypothesis is supported by the results in syngeneic mice in which the precursor frequency of antigen-bearing cells is increased by the adoptive transfer of CL4 T cells. In this case, a moderate capacity to eradicate tumors was demonstrated (40% protection). This capacity was enhanced further by using CL4 mice as tumor recipients where the number of antitumor effectors is even higher. In these mice, tumor was eradicated in 60% of recipients and was independent of the amount of antigen expressed by the tumor. But, although these data demonstrate that increased precursor frequency can increase the probability of tumor eradication, they also show that at superphysiological frequencies of precursor T cells, tumor rejection is not guaranteed.

**Tolerization Does Not Explain Why a Subpopulation of CL4 TCR Transgenic Mice Fails to Reject the Tumor.** What was surprising in our experiments was that 40% of the class I-restricted TCR transgenic mice failed to eradicate tumor, although the pool of effectors was similar in the entire experimental group. It was possible that the effector cells had been tolerized due to encounter with the tumor. The lack of costimulatory molecules on the MM cells could have led to anergy induction (30), or if these cells had been activated, they may have been deleted through clonal exhaustion (31). We found no evidence for tolerization because these cells could be isolated, in normal numbers, and subsequently protect naive recipients in a Winn...
assay. Also, after adoptive transfer into tumor-inoculated animals, CL4 peptide-reactive cells could still be identified.

Together these data are consistent with other studies that demonstrate that class I-restricted TCR transgenic mice have limited or no antitumor effects. Using lymphocytic choriomeningitis virus as a tumor antigen in an insulinoma model, it was demonstrated that, although the transgenic cells were able to destroy cells bearing lymphocytic choriomeningitis virus antigen, the destruction was transient, and both neoplastic and normal tissue remained relatively intact (26). The failure to fully eradicate the tumor was not due to tolerization of the CTLs but due to a failure to sustain an effective response. In a separate model, in which α-ketoglutarate dehydrogenase peptides in an Ld molecule are recognized by 2C TCR transgenic mice (32), it was shown that skin graft rejection could occur concomitantly with tumor engraftment, despite the presence of a shared target antigen (33). Again, there was no evidence for tolerance induction in the effector population. Although both these models are highly informative, they are limited by either the lack of defined class II epitopes or the use of allogeneic class I molecules, where host APC-transgenic T cell tumor antigen interactions are bypassed. As in our own model, a high precursor frequency of CTLs did not guarantee tumor eradication, and additional factors must be contributing to the variability in tumor growth.

One such factor may be the limited access of tumor-specific effectors to the tumor site (8). In our model, both the parental tumor line and the transfected lines show very few infiltrating lymphocytes in syngeneic recipients (34). Alternatively, the answer may lie in the nature of the tumor cell itself and its vulnerability as a target to CTL effectors. CTLs derived from the CL4 transgenic mice, although able to lyse standard targets, were unable to kill the AB1-HA cells unless the tumors were pretreated with IFN-γ. Thus, in vivo, a large number of effectors, in addition to their lytic function, may provide a sufficient source of local IFN-γ production at the tumor site to increase class I expression and promote effective CTL recognition. If such cytokine production is at a critical threshold, it is feasible that the process might not be 100% efficient, allowing tumor outgrowth in some animals. Interestingly, we have shown that the levels of IFN-γ within a solid tumor fall progressively during tumor growth (35).

A third alternative may be that tumor-specific CD4 cells can modulate CTL function and, when lacking, the destruction of tumor cells is relatively inefficient.

**Tumor-specific CD4 T Cells Are Unable to Directly Eradicate Tumors.** It is essential to understand the role of tumor-specific CD4 cells in generating antitumor immunity, especially for the rational design of vaccines that may need to incorporate class II epitopes. In
The past, the role of such CD4 cells has only been able to be implied by the isolation and use of long-term tumor-reactive CD4 clones, by global depletions of CD4 cells using antibody treatment, or by the ability of cytokines, such as IL-2, to enhance tumor eradication. These nonspecific approaches have led to variable results; some tumor studies have shown CD4 cells to be redundant (36), others have demonstrated that they are capable of direct antitumor activity (37), and others have shown that CD4 cells may act to suppress antitumor CD8 responses (38). Experiments to clarify the role of CD4 cells in tumor immunity clearly require the ability to directly identify and manipulate tumor-specific CD4 cells.

Our data demonstrate that tumor-specific class II restricted TCR transgenic mice fail to reject HA-transfected tumors and suggest that tumor-specific CD4 cells alone, even if present in large numbers, are not capable of causing tumor eradication. In the absence of class II expression on the tumor, this may not seem a surprising result but, despite the inability of the HNT cells to interact directly with the tumor cells, the AB1-HAHI line showed a significant delay in growth compared with the AB1-HA LO line. This delay was clearly due to immunological events because both transfectants grew at similar rates in T cell-deficient mice. The kinetics of tumor growth correlated with the level of HA expression, suggesting that the limited effect of the specific CD4 cells in modulating tumor growth was contingent upon reaching a threshold level of antigen expression, which is in contrast to the results with tumor-specific CD8 cells (see below). That the CD4 cells recognize tumor antigen in vivo is clear from the CFSE data, which demonstrate antigen-CD4 cell interactions in the draining LNs. These data clearly imply a role for the traffic of tumor antigen via APCs. This traffic is limited to the draining LNs and occurs in the absence of metastasizing tumor or up-regulation of class II on the tumor cells.

Tumor-specific CD4 Cells Synergize with Limiting CD8 Cells to Cause Tumor Eradication. CD4 cells are mostly considered as adjuvants to B cells and CD8 T cells through the provision of helper factors, although they have other potential roles in an immune response. In particular, CD4 cells have been shown to be the pathogenic effectors in some models of autoimmunity (39, 40), including a transgenic model of diabetes, which uses the same TCR transgenic mice as in our experiments.
The inability to demonstrate any direct role in the class II-restricted TCR transgenic mice and the variability of tumor growth in the class I-restricted TCR transgenics led us to investigate how CD4 cells might modulate CD8-mediated tumor eradication. We hypothesized that any effect of specific CD4 cells in our model would be more easily detected in an adoptive transfer approach using more limited numbers of CTL effectors. The addition of specific CD4 cells to the CTL pool produced 100% protection, substantially higher than in the intact CL4 transgenic mice or with either subpopulation alone. Importantly, the data also demonstrate that these CD4 cells must be tumor specific because naïve BALB/c CD4 cells could not substitute for the class II-restricted TCR transgenic cells.

How Do CD4 Cells Act to Improve Tumor Eradication by CD8 Cells? It is possible that CD4 cells may be obligatory at both the induction and effector phases of the immune response to ensure tumor eradication. CD4 cells act as helper cells for CTL function, and such a requirement for help in tumor immunity has been suggested in a number of vaccination studies in which IL-2 can replace CD4 function (15). CD4 cells may also augment the presentation of tumor antigens to CD8 cells by modifying the APCs (41–43) or impairing CD8 deletion (24). In our tumor studies, CD4 cells did not boost the CTL response by increasing the number of CD8 cells proliferating in the draining LNs. Nor, at least up to 3 days, did their presence lead to a change in the level of Fas expression on the CD8 cells. In our model, CD4 cells are not altering CD8 fate through changes in Fas, at least at relatively early time points. Thus, CD4 cells do not seem to be potentiating tumor eradication by influencing either of these processes.

Preliminary histological data show that when tumor-specific CD4 and CD8 cells are cotransferred into tumor-inoculated animals, there is a large infiltration of both subsets within the tumor. In the absence of CD4 cell

Fig. 8. CD4 cells do not enhance proliferation of CL4 cells in the draining LNs. CFSE-labeled CL4 cells (5 × 10⁶) were adoptively transferred into BALB/c mice inoculated with AB1-HA⁴⁴⁴ tumor alone (A and B) or together with 5 × 10⁶ HNT cells (C and D). After 72 h, lymphocytes from the draining (A and C) and nondraining (B and D) LNs were analyzed by flow cytometry. Profiles were gated on CFSE⁻ CD8⁺ cells. These results are representative of three experiments.

Fig. 9. CD4 cells do not act by decreasing Fas expression on the CD8 effectors. CFSE-labeled HNT and CL4 cells were transferred into animals as described in Fig. 7A. Three days later, CFSE labeled CL4 cells that had undergone different numbers of cell cycles in the draining LNs were analyzed for Fas expression. These were identified in a FACS dot-plot showing CFSE versus forward scatter. The histograms show the expression of Fas on the undivided cells (DO) and cells that have undergone six divisions (D6).
transfer, few, if any, lymphocytes, infiltrate the site, none of which are CD8+. These data imply that CD4 cells influence the trafficking of CD8 cells into the tumor. Once in the tumor, both CD4 and CD8 cells could contribute to produce IFN-γ, up-regulate class I, and allow CTL recognition of the target cells. This ability to modulate the tumor milieu is distinct from traditional helper functions, via IL-2, normally attributed to CD4 cells and may lead to increased tumor immunogenicity.

The data reported here establish the utility of this novel tumor model and demonstrate that the responses of both CD4 and CD8 cells can be analyzed. They also demonstrate the importance of a linkage between the CD4 and CD8 tumor-specific components of cellular immune response; specific CD4 cells activated by APCs that have processed the tumor antigen improve the capacity of limiting numbers of specific CD8 effector cells to eradicate solid tumors, either through direct help or indirectly by modifying the tumor milieu to produce up-regulation of class I, or possibly through APC modulation. This model provides an excellent basis for further dissection of the interactions that occur within the immune system to ensure tumor eradication.

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T-Cell Receptor Transgenic Analysis of Tumor-specific CD8 and CD4 Responses in the Eradication of Solid Tumors


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