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Tumor Antigens are Constitutively Presented in the Draining Lymph Nodes[1]

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Tumor growth is rarely associated with a strong specific CTL response, suggesting that the immune system is ignorant of the presence of tumor because the Ags are not readily available to or are sequestered from potential effector cells. We studied the in vivo activation of naive TCR transgenic hemagglutinin (HA)-specific CD8$^+$ T cells adoptively transferred into mice bearing HA-expressing tumor using 5,6-carboxy-succinimidyl-fluorescein-ester labeling, which allows the identification of proliferating HA-specific T cells. We demonstrate that tumor Ags are constitutively presented in the lymph nodes draining tumors and are powerfully mitogenic for responding T cells despite the absence of anti-tumor CTL responses. Importantly, this proliferative signal occurs throughout tumor growth and is still detectable 6 mo after tumor inoculation when tumor is not palpable. These results provide the first evidence that there is constitutive presentation of tumor Ags in draining lymph nodes. ***The Journal of Immunology*****, 1999, 162: 5838–5845.**

There are a number of mechanisms that have been proposed to explain how tumors evade immune destruction. T cell responses may occur but be ineffective due to a low precursor frequency of tumor-specific T cells that cannot expand to a level required to eradicate the tumor or because T cells may simply be unable to penetrate the tumor milieu (1). Alternatively, tumor-reactive T cells may enter the tumor and become nonresponsive due to a lack of expression of costimulatory molecules (2–5). T cells might fail to recognize tumor due to down-regulation of peptide/MHC expression on the tumor itself or APCs, which may, in turn, occur through the loss of the peptide transporter TAP or proteosome subunits (6–10). It is also possible that despite appropriate recognition and activation of tumor-specific T cells, the response remains ineffective due to immunosuppressive factors present in the tumor milieu such as TGF-β (11, 12) or IL-10 (13, 14), to anergy induction (15), or to the fact that they are induced to die (16–19). Implicit in each of these mechanisms there is a contention that host T cells can recognize tumor Ags.

In contrast, a number of investigators have suggested that the primary reason for the failure of host anti-tumor responses is ignorance, i.e., a failure of the host immune system to recognize tumor Ag in the induction phase. The concept of ignorance (20, 21) has gained wide acceptance by linking the apparent lack of effector cells with a failure in the induction and/or maintenance of an immune response. An assumption in the experiments that led to this concept is that the lack of measurable CTL activity correlates with T cell ignorance of tumor Ags. While CTL activity provides useful information that can correlate with tumor Ag recognition, it is still an indirect in vitro method of determining whether T cells have encountered Ag in vivo. To date, models in which tumor-specific T cell responses can be followed in vivo have been lacking.

By stably transfecting murine tumor lines with the influenza hemagglutinin (HA)[3] gene and studying the growth of this tumor in animals adoptively transferred with HA-specific TCR transgenic lymphocytes (i.e., tumor-specific lymphocytes) we have a very powerful model, in which HA is a tumor neo-Ag (it is expressed in a growing tumor yet is associated with weak CTL responses). Using this model it is possible to follow the response of class I-restricted tumor-specific T cells and the effects on the response of different levels of Ag expression. It is also possible to monitor where activation occurs, e.g., draining lymph node, other lymphoid organs, or tumor. This approach enables us to use T cells that have not undergone the substantial selection pressure exerted by cloning procedures. It also enables us to study the role of tumor-specific T cells, an approach that may be more informative than depleting gross T cell populations by Ab treatment or evaluation of tumor-infiltrating lymphocytes.

In this report we show that clonal expansion of tumor-specific T cells occurs in the lymph nodes that drain the tumor and that Ag is available to specific CD8$^+$ T cells throughout tumor growth, demonstrating that tumor Ag is not sequestered during tumor development. Importantly, we are able to demonstrate that Ag continues to be available even in the absence of detectable tumor and up to 6 mo after tumor inoculation.

Materials and Methods

Mice

BALB/c (H-2^d) mice were obtained from the Animal Resources Center (Canning Vale, Australia) and were maintained under standard conditions in the University Department of Medicine animal holding area. The TCR transgenic mouse line CL4 expresses a TCR recognizing an H-2K^d-restricted HA epitope (^{533}IYSTVASSL^{541}) (22) and was backcrossed for five

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[3] Abbreviations used in this paper: HA, hemagglutinin; MM, murine malignant mesothelioma; CFSE, 5,6-carboxy-succinimidyl-fluorescein-ester.

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generations onto the BALB/c genetic background. An HA-specific, MHC class II-restricted TCR transgenic line, termed HNT mice, has previously been described (23). CL4 transgenic progeny were typed by flow cytometry using mAbs anti-CD8-PE and anti-Vβ8.1-FITC; HNT mice were typed using anti-CD4-PE and anti-Vβ8.2,3-FITC (PharMingen, San Diego, CA). For all experiments female mice between 6–12 wk of age were used.

Murine tumor cell line

The derivation and characterization of the AB1 murine malignant mesothelioma (MM) cell line used in this study have been described previously (24). Essentially, the cells were generated by inoculating crocidolite asbestos i.p. into BALB/c mice, and the 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, Gaithersburg, MD) supplemented with 20 mM HEPES, 0.05 mM 2-ME, 100 U/ml penicillin (CSL, Melbourne, Australia), 50 μg/ml gentamicin (David Bull Laboratories, Mulgrave North, Australia), and 5% FCS (Life Technologies, Gaithersburg, MD). AB1 is a class I^+, class II^-, and poorly immunogenic tumor (25).

HA transfectant cells

Generation and characterization the of AB1 cells transfected with the influenza HA gene have been described (26). Briefly, the HA gene from the Mount Sinai strain of the PR8 influenza virus was subcloned into the β-actin expression vector (phβApr-neo, obtained from Dr. J. Allison, University of California-Berkeley). Transfected cells were selected by culture in medium containing the neomycin analogue geneticin (Life Technologies) at a final concentration of 400 μg/ml. The level of HA expression on transfected cells was measured by FACS analysis, using the biotinylated HA-specific mAb H18 (27) that was originally obtained from Dr. Walter Gerhard, The Wistar Institute (Philadelphia, PA). Two clones expressing different levels of HA by FACS analysis; one expressing relatively low levels of HA (AB1-HA^{low}) and one expressing relatively high levels of HA on the surface (AB1-HA^{high}) were then chosen for additional experiments.

RT-PCR analysis of lymph node cells for HA expression

Lymph node cells were resuspended in RNAzol B (Bresatec, Adelaide, Australia) and RNA purified as recommended by the manufacturer. One microgram of total RNA was reverse transcribed by standard methods and was amplified by PCR for 50 cycles using primers for HA, 5′-CAATT GGGGAAATGTAACATCGCCC-3′ and 5′-AGCTTTGGGTATGAGC CCTCCTTC-3′. The cDNA was coamplified with β-actin using primers 5′-CGTGACATTAAGGAGAA GCTGTGC-3′ and 5′-CTCAGGAGG AGCAATGATCTTGAT-3′. PCR products of 320 bp (HA) and 375 bp (β-actin) were observed after separation by electrophoresis in a 1.5% agarose gel. The limit of sensitivity of the PCR analysis was 10 cells.

Tumor inoculation

Tumor cells (1×10^6) were inoculated s.c. on one side of the ventral surface in the lower flank region. The draining lymph node was the inguinal node at the site of injection. The nondraining node was the contralateral inguinal node.

Ex vivo culture of lymph node cells

Lymph node cells were taken from BALB/c mice previously inoculated with AB1-HA tumor and were cultured in vitro for detection of tumor cells.

Generation of primary tumor-specific CTL

BALB/c mice were inoculated s.c. with AB1-HA tumor, and 7, 14, 21, and 28 days after inoculation, spleen cells were set up in vitro with specific peptide (1 μg/ml). Cells were harvested 5 days later, and CTL activity was determined.

CTL assay

Target cells were labeled with 150 μCi of ^{51}Cr for 90 min and were washed four times before use. Effector cells were added to corresponding targets at varying E:T cell ratios and were incubated at 37°C for 4 h. For cold target inhibition studies, labeled peptide-pulsed P815 targets were seeded at an E:T cell ratio of 50:1 with unlabeled peptide-pulsed P815, unpulsed P815, and YAC cells at various E:T cell ratios. These were incubated at 37°C for 4 h. After incubation, the supernatants were harvested, and chromium release was determined. The mean of triplicate samples was calculated, and the percent specific ^{51}Cr release was determined as follows: percentage of specific cytolysis = [(experimental ^{51}Cr release − control ^{51}Cr release)/(maximum ^{51}Cr release − control ^{51}Cr release)] × 100%, where experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from targets exposed to 5% Triton X-100.

5,6-Carboxy-succinimidyl-fluorescein-ester (CFSE) labeling of CL4 TCR transgenic lymph node cells

CFSE labeling was performed as previously described (28). Tumor-specific (HA-specific) lymph node cells from TCR transgenic, class I-restricted CL4 mice were resuspended in PBS at 1×10^7 cells/ml. For labeling, 2 μl of a CFSE (Molecular Probes, Eugene, OR) stock solution (5 mM in DMSO) were incubated with 10 ml of cells (at 10^7/ml) in RPMI medium without FCS for 10 min at room temperature. Cells were washed through FCS four times and then were resuspended in PBS before i.v. injecting 1×10^7 cells into recipients. In all experiments CFSE-labeled cells were recovered 3 days postadoptive transfer and were analyzed by FACS analysis. CSFE cells were adoptively transferred at time points that meant that the degree of proliferation was analyzed on days 7, 14, and 28 days posttumor inoculation or at subsequent longer time points relevant to the individual experiment as detailed in the text and figures.

FACS analysis

Lymph node or spleen cells were stained for three-color FACS analysis using the following mAbs: PE-conjugated anti-CD44 (IM7), biotinylated anti-CD8a (53-6.7), and anti-CD4 (RM4-5; PharMingen). Biotin-labeled Abs were detected with streptavidin (Zymed, South San Francisco, CA). Analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest software. For analysis of CFSE-labeled cells, 50,000 events were collected and analyzed using the modFit LT cell cycle analysis software (Verity Software House, Topsham, ME).

Proliferation assay

BALB/c mice were immunized with either 1×10^6 AB1-HA^{high} or AB1 parental tumor cells s.c and injected i.v. with 1×10^7 CL4 or CL4 plus HNT lymph node cells. Draining and nondraining lymph nodes were isolated 2 and 4 wk after the adoptive transfer and were treated with a 1/3 dilution of 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, Mequon, WI) in RPMI with 10% FCS for 30 min at 37°C. B cells were removed by negative selection with anti-B220 mAbs (RA3-6B2, PharMingen) followed by bead separation using beads coated with sheep anti-rat IgG (Dynal, Oslo, Norway). Cells were then washed twice, and purity was checked by FACS analysis using FITC- and PE-conjugated anti-CD8, anti-CD4, or anti-B220 (PharMingen). Analysis was performed on a FACScan (Becton Dickinson) using CellQuest software. Cells were plated in triplicate in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at a concentration of 1×10^6 cells/well in 200 μl RPMI and 10% FCS containing penicillin/streptomycin. These cells were cultivated for 48 h with or without the CL4 peptide, and [^{3}H]thymidine was added 12–15 h before harvesting. To determine the sensitivity of this assay in detecting the number of CL4-positive cells, CL4 lymph node cells were titrated in the presence of nontransgenic lymph node T cells ranging from 0–50% of the total number of cells.

Results

Tumor growth is associated with a poor, transient generation of peptide-specific CTL

The murine malignant mesothelioma cell line AB1 was transfected with the HA gene, and two clones that had relatively high (AB1-HA^{high}) or low (AB1-HA^{low}) levels of surface HA expression were chosen for experimental work (Fig. 1). BALB/c mice were injected with AB1-HA^{high} tumor cells, and spleen cells were removed 7, 14, 21, or 28 days later for analysis of CTL function. Low levels of lytic activity (<5% specific lysis against the tumor itself; i.e., AB1-HA^{high} targets) were detected 7 days posttumor inoculation (Fig. 2*A*); the data shown represent the best level of CTL activity detected in more than three independent experiments. At subsequent time points CTL activity was not detected at all, nor did peptide pulsing of the targets with the HA K^d-restricted peptide enhance the lysis of the AB1 cells (Fig. 2*A*). This lack of CTL activity was not due to an inherent insusceptibility of these targets to CTL-mediated lysis, as CTLs derived from the HA-specific

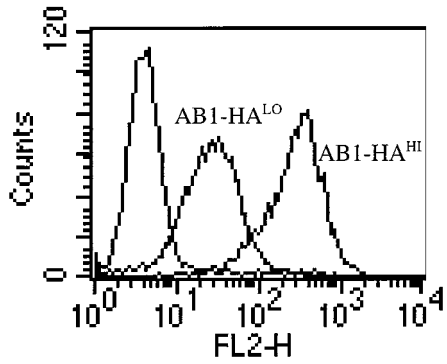

FIGURE 1. Relative level of surface HA expression on AB1 transfectants. FACS analysis demonstrating the relative level of surface HA expression on the AB1 transfectants AB1-HA^{low} and AB1-HA^{high}.

TCR transgenic mice are able to lyse AB1-HA tumor cells (>80% specific lysis), nor was it due to loss of HA expression during tumor growth (26). However, a transient low level CTL response was detected when peptide-pulsed P815 cells were used as targets. Maximal CTL activity (<15% specific lysis) occurred on day 7 after tumor injection and diminished to <5% lysis by day 28 (Fig. 2*B*). Over this period of time, tumors continued to grow in all AB1-HA^{high}-inoculated animals (Fig. 2*D*). The weak CTL responses derived from mice inoculated with tumor contrasted with that obtained when mice were inoculated with influenza virus, in which lysis of HA peptide-pulsed targets ranges from 40–80% and did not decline over 28 days (Fig. 2*C*).

Tumor-specific CD8$^+$ T cells proliferate in lymph nodes draining the AB1-HA^{high} tumor

The deficiency of the CTL response to AB1-HA targets and its transience may have been due to either the sequestration of tumor Ag from the immune system or a decline in Ag availability over time. To investigate these possibilities an adoptive transfer model was used in which lymph node cells from the CL4 TCR transgenic mice, a class I-restricted HA-specific TCR transgenic line (22), were labeled with the fluorescent dye CFSE and transferred into AB1-HA or AB1 inoculated BALB/c mice (1×10^6 cells s.c.). Three days after the transfer of the CL4 cells into the various recipients, lymphoid tissues were removed and prepared for FACS analysis. Therefore, in these studies the CL4 cells represent a source of CD8$^+$ T cells specific for the nominal tumor Ag HA. When CFSE-labeled cells divide, the progeny retain approximately half the original fluorescence of the parental cell and so on. This decay in fluorescence intensity is recorded as separate peaks on a FACS histogram in which daughter cells can be detected for up to seven divisions, after which the fluorescence is not distinguishable from background.

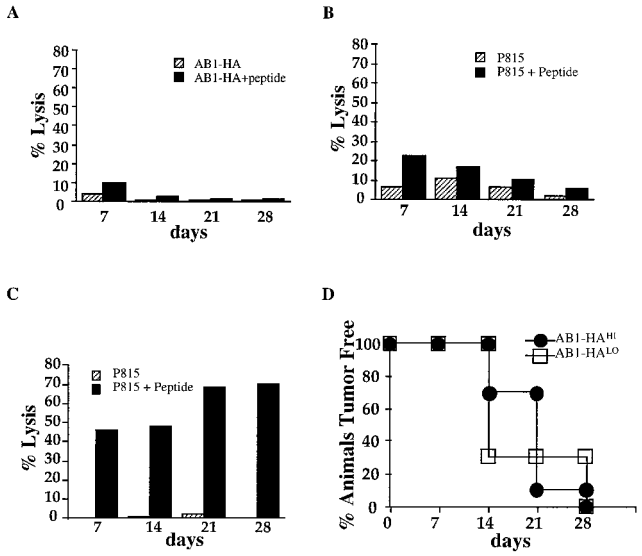

FIGURE 2. Generation of a transient tumor-specific CTL. HA-specific CTL activity in BALB/c recipients was assayed at multiple time points post-AB1-HA^{high} inoculation and was tested against AB1-HA^{high} targets with or without HA peptide (*A*) and against P815 with or without HA peptide (*B*). This activity can be compared with that obtained from influenza-primed mice against P815 with or without HA peptide (*C*). Ten BALB/c mice injected s.c. with AB1-HA^{high} or AB1-HA^{low} were monitored for tumor growth over the same time period as the CTL analyses (*D*). The results shown here are representative of three such experiments.

Proliferating CL4 cells represented the majority of the CFSE-labeled cells (93%) within the lymph node draining the AB1-HA^{high} tumor when analyzed 3 days after adoptive transfer, i.e., on day 7 after tumor injection (Fig. 3*A*). In fact, proliferation of CL4 cells could be detected as early as day 3. At both time points tumor growth was not detectable by palpation. In contrast, CL4 cells did not proliferate in the lymph node draining the AB1-HA^{low} tumor, the lymph node draining the AB1 parental tumor (Fig. 3*A*), or the nondraining lymph nodes from any tumor-bearing animal (Fig. 3*B*). These data correlate well with the overall percentage of CFSE-labeled cells found within these lymph nodes at the time of analysis. In draining nodes CFSE$^+$ cells represented 2–3% of the total cells. In nondraining lymph nodes or nodes isolated from nontumor-bearing animals CFSE$^+$ cells represented ≤1% of the total cells (data not shown). We also examined transferred cells for expression of CD44 (Pgp1), which is increased upon T cell activation. In concordance with the proliferation data, CL4 cells from the draining lymph nodes of AB1-HA^{high} inoculated animals showed an increase in CD44 expression (Fig. 3*C*), whereas there was no change in the CD44 expression on CL4 cells isolated from the draining lymph nodes of AB1-HA^{low} or AB1 parental tumor-bearing animals. In addition, there was no increase in the expression of CD44 on cells obtained from the nondraining lymph nodes of animals inoculated with AB1-HA^{high}, AB1-HA^{low}, or AB1 parental tumor (data not shown). Significantly, there was no evidence of tumor cells in the draining lymph nodes as determined by in vitro culture of the draining lymph node or by PCR analysis to detect HA gene expression (data not shown).

When the level of tumor Ag expression is low, a larger tumor burden is required for CD8$^+$ T cells to proliferate

The experiments described above were extended to investigate whether the tumor burden, and potentially the tumor Ag concentration, affected the capacity of CL4 cells to proliferate to tumor Ag. Indeed, this was found to be the case, as, in contrast to day 7, on day 14 after tumor inoculation, CL4 cells were able to proliferate in the draining lymph nodes of both AB1-HA^{high}- and AB1-HA^{low}-bearing animals (Fig. 4*A*). This difference between AB1-HA^{high} and AB1-HA^{low} tumors in causing in vivo Ag-specific proliferation was not due to differences in the rate of tumor growth (Fig. 2*D*) (26). Again, there was no proliferation of CL4 cells in the draining lymph node of AB1 parental tumor-bearing animals, confirming that the proliferation observed was not due to nonspecific factors (Fig. 4*A*). As for day 7, proliferating CL4 cells were never detected in nondraining lymph nodes of any tumor-bearing animal (data not shown).

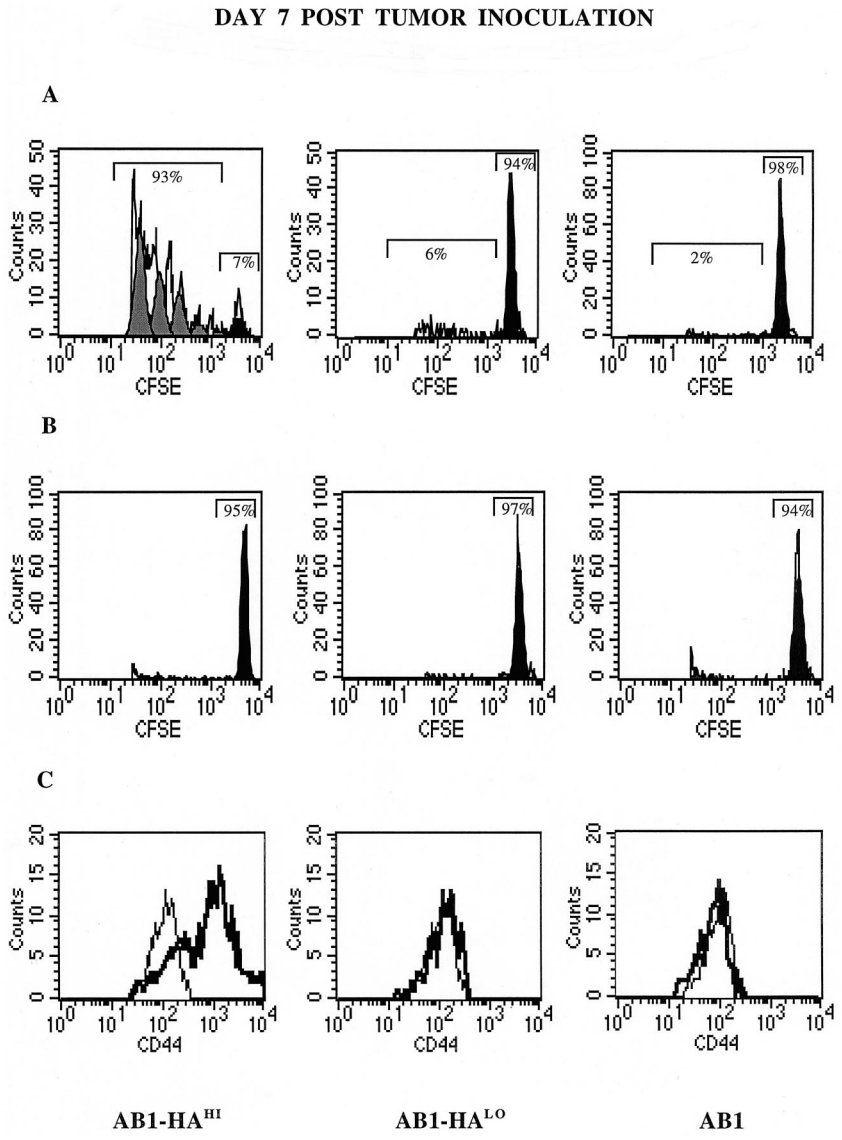

FIGURE 3. Tumor-specific CD8$^+$ T cells proliferate, and CD44 staining is increased in the draining lymph nodes of AB1-HA^{high} animals 7 days posttumor inoculation. FACS analysis of CFSE-labeled, class I-restricted, HA-specific TCR transgenic T cells that had been adoptively transferred into AB1-HA^{high}, AB1-HA^{low}, or AB1 tumor-bearing mice on day 4 posttumor inoculation was performed. These cells were isolated from the tumor-draining lymph node (*A*) and the nondraining lymph node (*B*) 3 days posttransfer. Cells from the draining lymph node were also stained for CD44 expression (*C*). All profiles obtained were gated on CD8$^+$ T cells. These results are representative of five such experiments consisting of three mice per group.

Tumor Ag is available in the lymph nodes draining the tumor throughout tumor growth

In extended kinetic experiments, CL4 cells were shown to proliferate after isolation in mice inoculated with AB1-HA tumor 21 and 28 days previously (Fig. 4, *B* and *C*). At this last time point the majority of animals were euthanized due to the size of the tumor. Even at this advanced stage of disease, proliferation of CL4 cells was not observed in the draining lymph nodes of AB1 tumor-bearing animals (Fig. 4, *B* and *C*) or in the nondraining lymph nodes of any tumor-bearing animal (data not shown).

Tumor Ag appears to be retained long term in the lymph node draining the site of tumor injection

Of 60 BALB/c mice inoculated with AB1-HA^{high}, three mice did not develop tumor. Transferred CL4 cells were still able to proliferate substantially within the lymph nodes draining the site of tumor injection at 32, 72, and 179 days posttumor inoculation (Fig. 5, *A–C*) even though there was no evidence of tumor growth by palpation or macroscopic examination. Such proliferation did not occur in the nondraining lymph nodes (Fig. 5, *D–F*).

Proliferating tumor-specific CTLs do not survive in vivo unless tumor-specific CD4 $^+$ T cells are present

These experiments clearly show that tumor Ags are constitutively available to the immune system even in the absence of detectable CTL responses. Thus, the failure to detect effective responses is due to post-Ag recognition events. One potential point at which the immune system may fail is in the ability to maintain effector cell function. In fact, in an autoimmune model it has been demonstrated that effective effector function correlates with the survival of cross-primed CD8 effectors, and better survival is associated with the concomitant presence of CD4 cells (28). To test whether tumor-specific CD8$^+$ T cells survive in vivo after adoptive transfer, mice were inoculated s.c. with either AB1-HA^{high} or parental AB1 tumor at the same time as transferring CD8-purified CL4 cells. Because there is no clonotype Ab available for the CL4 transgenic T cells we approximated the survival of CL4 cells by determining CL4 peptide reactivity. Lymph node cells were isolated at 2 and 4 wk posttumor inoculation and T cell transfer and were tested for CL4-peptide reactive cells in a proliferation assay (Fig. 6). While CL4 cells could be retrieved at 2 wk postinoculation, in the absence of specific Ag these represented $<0.1\%$ of the

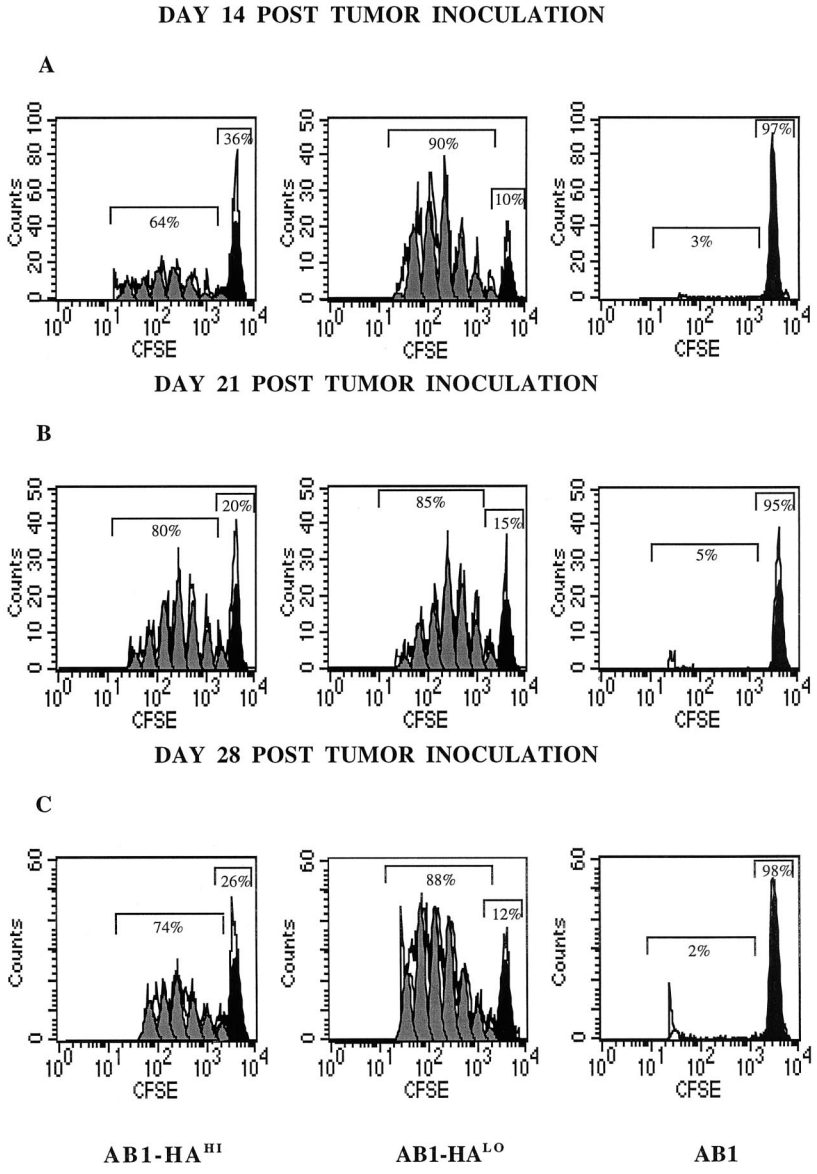

FIGURE 4. A threshold level of Ag appears to be required for $CD8^+$ T cells to proliferate, but Ag presentation is subsequently maintained throughout the period of tumor growth. FACS profiles of CFSE-labeled, tumor-specific $CD8^+$ T cells from the draining lymph nodes of BALB/c mice injected s.c. with AB1-HA^{high}, AB1-HA^{low}, or the parental cell line AB1. CFSE cells were reisolated from the draining lymph nodes 14 (*A*), 21 (*B*), and 28 (*C*) days posttumor inoculation after having been adoptively transferred 3 days previously. This experiment was performed twice with three or four mice per group.

lymph node population and by 4 wk were undetectable (Fig. 6). In the presence of Ag, CL4 cells were still detectable at 2 wk (~0.1% of total cells as determined by the proliferation assay), presumably due to continued proliferation of residual cells but, again, were completely undetectable by 4 wk.

To test the effect of $CD4^+$ T cells on CL4 T cell survival, we added CD4-purified HNT cells, HA-specific, class II-restricted TCR transgenic T cells (23), at the time of CL4 cell transfer in the adoptive transfer. Unlike when CL4 cells are transferred alone, a decline in CL4 reactivity over time was not evident when HNT cells were cotransferred (Fig. 6). In fact, the complete opposite was true, CL4 peptide reactivity increased over the 4 wk of observation.

Discussion

Tumor-specific $CD8^+$ T cells are not ignorant of the presence of tumor Ag

Our data demonstrate that tumor Ag is available to tumor-specific T cells even in the absence of an ability to generate effective antitumor CTL activity. This process is clearly Ag specific, as no proliferation was observed when these T cells were transferred into animals bearing the identical tumor but without the specific Ag. Interestingly, this appeared to be a very strong signal as proliferating cells represented >80% of the adoptively transferred T cells within the draining lymph node.

The mechanism by which the tumor Ag reaches the draining lymph node is not specifically addressed in these experiments; however, there was no evidence that tumor cells were present within the draining lymph node based on histology, tissue culture, and PCR, minimizing the possibility that direct tumor-T cell interactions lead to Ag-driven proliferation. While there may be release of intact HA protein with subsequent transport to the lymph node, we believe that it is most likely that the presentation of the HA Ag is occurring via professional APCs, which are likely to have trafficked from within the tumor. This is based on the observations that there are large numbers of macrophages, with some dendritic cells, within the tumor tissue, which can, when removed from the tumor tissue, present Ag efficiently (our unpublished observations) and that HA expressed in the tumor as class I peptide only can induce similar proliferation (our manuscript in preparation). Such cross-presentation of Ags via host APCs has been directly shown to occur in normal tissues (29) and in tumors when effective CTLs can be induced (30). While it could be argued that

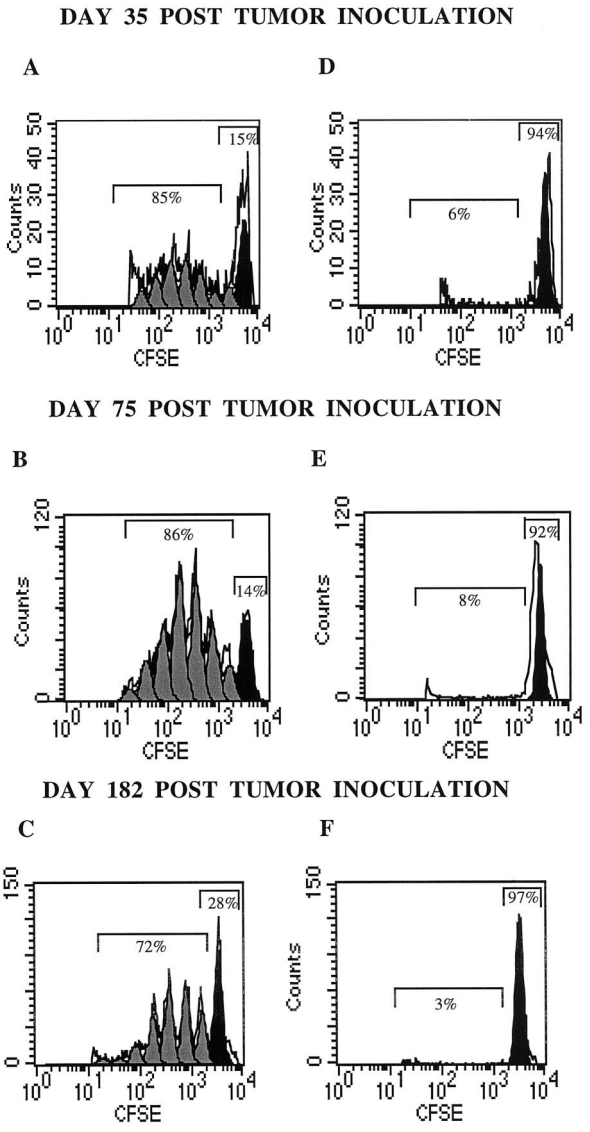

FIGURE 5. Proliferation of tumor-specific $CD8^+$ T cells still occurs even if there is no obvious tumor. BALB/c mice were inoculated with AB1-HA^{high}, and three mice did not develop tumor by 32, 72, and 179 days posttumor inoculation, respectively. CFSE-labeled tumor-specific cells were adoptively transferred i.v. into these mice, reisolated from the draining (*A–C*) and nondraining lymph nodes (*D–F*) 3 days postadoptive transfer of the CFSE-labeled cells, and analyzed by FACS.

these T cells are induced to proliferate elsewhere and are only "seen" in the draining lymph node, we believe that this is unlikely because there is no evidence of T cell proliferation in other lymphoid organs, for example in the spleen, nondraining lymph nodes, or in the tumor itself.

At least two recent studies using TCR transgenic mice to investigate antitumor responses, both using lymphocytic choriomeningitis virus as a model tumor Ag, have reported that the immune system is ignorant of tumor Ag expression, and only exogenous priming, by either viral infection or Ag-pulsed dendritic cell vaccination can effective immune responses be initiated (21, 31). Spieser et al. used tumor destruction and CTL readouts as the means by which "ignorance" was assessed. Our experiments show that these criteria do not necessarily indicate that tumor Ag is not accessible to the immune system. Hermans et al. investigated the activation phenotype of transferred tumor-specific TCR transgenic

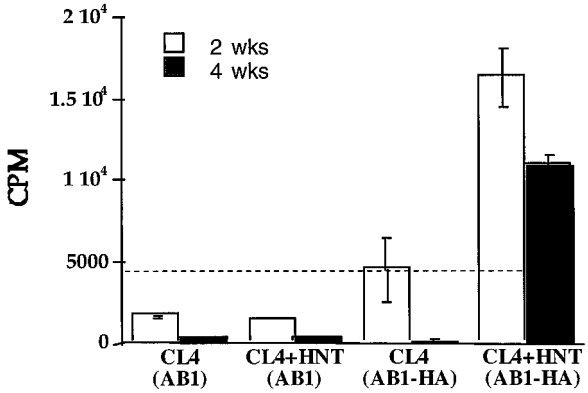

FIGURE 6. Cotransfer of tumor-specific $CD4^+$ T cells maintains CL4 cells in tumor-inoculated animals. BALB/c mice were inoculated with AB1-HA^{high} and were adoptively transferred with CD8-purified CL4 cells with or without CD4-purified HNT cells. At 2 and 4 wk posttumor inoculation draining lymph nodes were isolated, and cells were tested for reactivity toward the CL4 peptide. The dotted line represents the proliferation obtained when CL4 cells represent 0.1% of the total cells. This experiment is one of two similar experiments performed.

T cells and found no evidence of tumor Ag recognition by this criterion. This observation may be explained by the fact that they investigated transferred T cells derived from splenocyte preparations. In our model, tumor Ag recognition occurred only in the draining lymph node of tumor-inoculated animals; even extensive tumor growth (>100 mm^2) did not lead to tumor Ag trafficking to other lymph nodes or the spleen.

Tumor-specific $CD8^+$ T cells proliferate in draining lymph nodes even when tumor Ag concentration is relatively low

The finding that tumor cells with low HA expression induced proliferation of HA-specific T cells in the draining lymph node with delayed kinetics compared with those induced by tumor expressing higher levels of HA was not due to differences in the growth rates of these cell lines. Perhaps the simplest explanation is that a critical threshold of Ag is required to observe proliferation, and with relatively low Ags concentrations, there is a type of linear relationship between tumor size and Ag concentration. Alternatively, it may be that more Ag is available because larger tumors are more necrotic, and that this process is responsible for the release of Ag and its subsequent presentation to tumor-specific T cells. Experiments to attempt to differentiate between these two possibilities are currently being undertaken. Ultimately, however, the Ag concentration is not limiting in this system. However, it is important to note that even a relatively low HA concentration is a strong signal for T cell proliferation, with >90% of specific T cells on day 14 being in the proliferating pool. These data imply that the process of tumor Ag recognition within the draining lymph node is highly efficient.

Presentation of tumor Ags is not transient but continues throughout tumor growth

As the CTL response to tumor Ag was weak and transient, fading after 2–3 wk, it could be reasoned that this reflects transient Ag recognition, perhaps temporarily increased by the inflammation associated with s.c. injection of tumor cells. Yet our observation that even after 1 mo when the tumors had grown to a substantial size, with no evidence of inflammatory infiltration, tumor-specific T cells within the draining lymph node could receive strong mitogenic signals clearly shows that tumor Ags are continuously presented to the immune system. This phenomenon occurred regardless of the level of tumor Ag expression. Perhaps surprisingly,

even after 1 mo there was no evidence of tumor-specific T cell proliferation within nondraining lymph nodes or other sites, implying that Ag distribution and/or traffic of Ag-bearing APCs are restricted to the draining lymph node.

These results parallel those seen by Kurts et al. in their autoimmune model in which the nominal Ag OVA is constitutively expressed in the pancreas and kidneys (29). In these experiments there was no obvious organ damage or T cell infiltration, yet adoptively transferred OVA-specific T cells underwent strong proliferation in draining lymph nodes in a fashion similar to that observed in our own experiments. While these investigators were using a different Ag, a different animal strain, and a nontumor situation, the strong similarity between the results suggests that this process may be universal, i.e., that $CD8^+$ T cells continuously recognize tissue Ags in draining lymph nodes and undergo proliferation.

Tumor Ag is available to the immune system over an extended period of time

We were surprised to find evidence of strong tumor Ag-specific proliferation in lymph nodes draining sites of tumor injection but without apparent tumor growth as much as 6 mo after tumor inoculation. At first glance, this result may appear incongruous with the observation that there is a threshold of Ag required to obtain significant proliferation of tumor-specific cells. In fact, this is not the case. AB1-HA^{low} did not induce proliferation until the tumor was palpable, whereas AB1-HA^{high} induced proliferation up to 2 wk before the tumor growth was obvious, consistent with a threshold effect. In the experiments in which mice had apparently rejected the tumor, the mice had been inoculated with the AB1-HA^{high} transfectant. These data can be reconciled by two possible explanations. Firstly, the tumor cells may have remained quiescent yet viable in the s.c. site, releasing sufficient Ag to induce local T cell proliferation in the draining lymph node. This is consistent with experimental tumor models in which, under some circumstances, tumors remain quiescent for some months before emerging, for example in some animals transfected or injected with IL-12 (32) or constitutively expressing B7-1 (5). Secondly, the tumor may have been rejected, and the continuous presentation of tumor Ag in the lymph node could reflect the persistence of Ag-loaded APCs. The long term persistence of Ag has been demonstrated in other models, particularly those investigating the mechanisms of T cell memory (33–35).

The fate of tumor-specific $CD8^+$ T cells

Given that there is continuous tumor Ag presentation, what is the fate of the tumor-specific cells that encounter their Ag? From the above assertions the following general statements can be made. Tumor-specific $CD8^+$ T cells enter the lymph nodes but only encounter Ag in the nodes draining the site of tumor growth. This leads to several rounds of proliferation; most of it apparently occurs in the node, but the number of specific T cells eventually declines over several weeks in the absence of specific $CD4^+$ T cells. CTL generation occurs only during the first week following tumor inoculation and declines thereafter. These observations could be explained by anergy induction, the generation of specific suppression, or T cell deletion. We believe that $CD8^+$ T cell deletion is the most likely explanation because 1) similar effects on Ag-specific cells have been observed in studies of tolerance to nontumor Ags (36); and 2) CD4 help, such as the production of IL-2, provides survival signals for $CD8^+$ T cells. In the rat insulin promoter-OVA transgenic model, cross-presentation of tissue Ags occurs in the draining lymph node, but T cell numbers fall in the absence of sufficient $CD4^+$ T cell help (36). In tumor protection experiments using the AB1-HA tumor and the adoptive transfer of TCR transgenic T cells we have shown that both class I- and class II-restricted tumor-specific T cells are required for effective tumor eradication, and HNT (CD4) tumor-specific T cells do not prolong the life span of the CL4 (CD8) T cells by increasing the initial expansion of CL4 T cells (26). We are currently undertaking a number of experiments to evaluate how tumor-specific $CD4^+$ T cells influence the fate of the proliferating $CD8^+$ T cells and determine the mechanism by which $CD4^+$ T cells synergize with $CD8^+$ T cells to prevent tumor growth. Regardless of the mechanism, the failure to generate CTLs among the tumor-specific $CD8^+$ T cell population that can undergo strong, specific proliferation in response to tumor Ag recognition places the defect in host antitumor responses at a later point in the pathway of the immune process.

The implications of these findings for tumor immunology and immunotherapy

These findings have a number of important implications for tumor immunology. Firstly, they provide evidence that lymph nodes draining tumor sites, whether they be early, advanced, or occult tumor sites, are important potential sources of ongoing stimulation of tumor-specific T cells. It is common to find enlargement of lymph nodes draining human tumor sites in the absence of any evidence of invasion of those lymph nodes by tumor cells. The requirement for surgical removal of such lymph nodes has been and remains a contentious issue in cancer management (37). The data in this study suggest that it is impossible to assume that any swollen lymph node draining a tumor should be removed. In fact, if the additional factors required to translate this proliferative T cell response into an effective tumor-destructive response can be identified, it may be important to leave noninvolved draining lymph nodes intact. Secondly, these data illustrate that there is more to the monitoring of antitumor responses than simply the generation of measurable CTLs. Attempts to generate a strong antitumor response will need to focus on those factors that drive this proliferative phase into a tumor-destructive response. This is particularly important for the generation of tumor vaccines and the development of antitumor immunotherapy approaches. For example, these studies will enable us to determine which Ag stimulates or fails to stimulate tumor-specific T cell proliferation regionally. It may be that any focus on class I epitopes is too narrow, and the development of strong class II epitope strategies may be equally appropriate. Our approach can also elucidate the type and nature of cells required for adoptive immunotherapy experiments (e.g., $CD4^+$ Th cells rather than simply $CD8^+$ T cells) and may help to identify optimal cytokine regimens (e.g., those that help $CD8^+$ T cell responses develop into effective responses rather than simply inducing $CD8^+$ T cell proliferation). Thirdly, this approach may help to elucidate the biology of tumor metastasis. Recent studies using PCR demonstrated that there are variable numbers of tumor cells in the circulation of many tumor-bearing hosts (38). As proliferation in draining lymph nodes is a very sensitive technique for determining the presence of regional tumor Ags, it may be possible to use this technology to screen lymph nodes draining multiple sites in a host to determine the nature and kinetics of tumor cell distribution throughout the body. Fourthly, the sensitivity of this approach will enable us to determine the effect of conventional treatment approaches, such as surgery, chemotherapy, and radiotherapy, on specific antitumor responses.

In conclusion, this is the first demonstration that there is constitutive presentation of tumor Ags to tumor-specific T cells in draining lymph nodes that are capable of inducing proliferation and activation of specific T cells. This occurs continuously during tumor growth, even if the tumor Ag concentration is relatively low

and does not always lead to CTL generation. Surprisingly, this process is manifest when there is no obvious tumor. We propose that the lack of an effective immune response to tumors is not due to an inability to recognize tumor Ag but to a failure of the immune response to develop and persist following Ag recognition. Overall, these findings have major implications for further studies of tumor immunology and immunotherapy.

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