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Activation of Low Avidity CTL Specific for a Self Epitope Results in Tumor Rejection But Not Autoimmunity[1]

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To determine how self-tolerance can alter the ability of the immune system to respond against tumor-associated Ags that are also expressed by normal tissue, we designed experiments in which the same protein was expressed both as a tumor Ag and as a transgene product. Unlike conventional BALB/c mice that rejected renal carcinoma cells transfected with the influenza virus hemagglutinin (Renca-HA), transgenic mice that are tolerant of HA due to its expression as a self-Ag on pancreatic islet β cells, (Ins-HA mice) supported progressive growth of these tumor cells. However, when Ins-HA mice were immunized with a recombinant strain of vaccinia virus expressing the dominant H-2K^d peptide epitope of HA before receiving Renca-HA cells, they too were able to reject the tumor cells. Rejection of Renca-HA cells by immunized Ins-HA mice was found to be associated with the generation of CTL having much lower avidity for target cells presenting the K^{d}HA epitope than CTL from immunized conventional BALB/c mice. Significantly, we show that self-tolerance to the HA Ag is quantitative rather then absolute, and that vaccination of Ins-HA mice can activate low avidity K^{d}HA-specific CD8$^+$ T cells that are able to reject tumor cells expressing high levels of HA, yet these mice remain tolerant of pancreatic islet β cells expressing HA. ***The Journal of Immunology,*** **1998, 160: 643–651.**

One of the major goals of immunotherapy is to generate and direct CTL that can effectively eliminate tumor cells. Much research has focused upon identifying and characterizing proteins expressed by tumor cells that may serve as potential tumor-specific Ags for recognition by CTL (1). Some of the most promising candidates represent conventional cellular proteins that are expressed in both normal and transformed cells (2–13). Accordingly, the induction of self-tolerance could impose major limitations upon the availability of CTL with specificity for tumor-associated Ags (14–16).

Studies with transgenic mice expressing model proteins as self Ags have shown that T cells with specificity for self proteins can be demonstrated within the peripheral T cell pool (17–27). Although in many cases this may be due to the absence of thymic expression of the transgene, thereby precluding thymic deletion (28–31), often potentially autoreactive T cells manage to escape deletion by virtue of their lower avidity for self Ag even when the transgene product is expressed in, or is available to the thymus (19, 21, 24, 25, 27, 32). Thus, as tolerance does not necessarily result in the elimination of all self-reactive T cells, efforts to mobilize those residual cells within the repertoire may provide populations of CTL that are capable of destroying tumor cells expressing self Ags.

Indeed, several recent studies have demonstrated variable success in impeding the growth of certain tumors by immunization with self epitopes that are also tumor-associated Ags (33–36). Even though T cell responsiveness toward the tumor Ag under investigation was exhibited, no information was available concerning the degree to which self-tolerance may have altered the T cell response repertoire of the host. Thus, to assess the impact of self-tolerance on tumor immunotherapy, it would be useful to compare T cell responses by individuals that express the tumor Ag as self protein with those that do not.

To study the effects of tolerance upon the host's ability to eliminate tumor cells expressing self epitopes, we have explored the immune response to the influenza virus hemagglutinin (HA)[3] expressed both as a tumor Ag in renal carcinoma cells, and as a tissue-specific self Ag in Ins-HA transgenic mice that express HA on pancreatic islet β cells. As demonstrated previously, these mice are tolerant of the β cell-expressed HA, even after immunization with influenza virus (37). In this current study, we examine the impact of such tolerance upon the growth in these mice of the HA-expressing renal carcinoma cell line.

Materials and Methods

Mice

BALB/c mice were purchased from the breeding colony of The Scripps Research Institute (TSRI, La Jolla, CA). Ins-HA transgenic mice (37) and Clone-4 TCR transgenic mice (38) were generated and characterized as previously described. Each line was back-crossed for at least eight generations with BALB/c mice. All mice were bred and maintained under specific pathogen-free conditions in TSRI vivarium. All experimental procedures were conducted according to the guidelines laid out in National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Cells

The renal carcinoma cell line Renca (39) was originally provided by Dr. Robert Wiltrout (National Cancer Institute, Frederick, MD). Renca-HA

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[3] Abbreviations used in this paper: HA, hemagglutinin; CTLp, cytotoxic T lymphocyte precursor; Ins, insulin; NP, nucleoprotein; DAB, diaminobenzidine; Vacc, vaccinia virus; WT, wild-type.

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was generated by calcium phosphate-mediated plasmid transfection with the construct pIHA, which encodes the HA molecule of the influenza virus A/PR/8/34 (H1N1). Transfection was performed using a Stratagene kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Transfectants were selected in 400 mg/ml of the neomycin analogue, G418 (Life Technologies, Gaithersburg, MD). Drug-resistant cells were sorted for HA expression by staining with the mAb H18, and collecting $>10^5$ HA-bright cells per sort to maintain polyclonality. Sorted cells were expanded and resorted a total of five times. Both Renca and Renca-HA cells were maintained in our laboratory by weekly passage in RPMI 1640 medium containing 10% v/v FCS, 25 mM HEPES, 2 mM glutamine, 5×10^{-5} M β-mercaptoethanol, and 50 mg/ml gentamicin (complete RPMI). Renca-HA cells were grown in complete RPMI supplemented with 200 mg/ml of G418. The SV40-transformed H-2^d cell line, B10.D2, was used as a target cell in ^{51}Cr release assays. It was obtained from Dr. Barbara Knowles, University of Pennsylvania (Philadelphia).

Viruses

Influenza virus A/PR/8/34/(H1N1) was grown in the allantoic cavity of 10- to 11-day-old hen's eggs. Upon isolation, the allantoic fluid was titered for hemagglutination using chick RBC and stored in 1-ml aliquots at −70°C. Wild-type vaccinia virus (Vacc-WT), and the recombinant vaccinia viruses expressing either the whole HA protein (Vacc-HA), or the H-2K^d-restricted HA peptide, IYSTVASSL, (HA(M518–526)-Vacc-K^{d}HA, were kindly provided by Drs. Jack R. Bennink and Jonathan Yewdell from National Institutes of Health.

Peptide

Influenza virus A/PR/8/34 (H1N1) HA peptide (518–526) (sequence: IYS TVASSL) (40) and nucleoprotein (NP) peptide (sequence: TYQRTRALV) were synthesized by TSRI core facility using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA).

Immunization

Six- to eight-week-old mice were injected i.p. with virus. Mice were given 1200 HA units of influenza virus A/PR/8 in the form of allantoic fluid, or 10^8 plaque-forming units of Vacc-K^{d}HA in PBS.

Flow cytometry

Single cell suspension of Renca and Renca-HA cells was prepared, and 1×10^6 cells were incubated for 20 min on ice with the mouse anti-HA Ab 37/38. Cells were washed three times in PBS containing 1% w/v BSA (Sigma Chemical Co., St. Louis, MO) and 0.02% w/v sodium azide. Cells were then incubated for 20 min on ice with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Avondale, PA). All cells were analyzed with a FACScan and CELLQuest software (Becton Dickinson, Mountain View, CA).

Production of effector CTL

Mice immunized with either influenza virus A/PR/8 or Vacc-K^{d}HA were killed after 3 wk, and splenocytes were seeded into 24-well tissue culture plates at 6×10^6 cells/well in 1 ml complete RPMI. APCs were prepared as follows: splenocytes from BALB/c mice were irradiated with 3000 rad and then pulsed for 1 h with either 5 μg/ml of K^{d}HA peptide or 1200 HA units of influenza virus A/PR/8 in 1 ml complete RPMI without FCS. After washing three times in complete RPMI to remove unbound virus/peptide, 1 ml containing 6×10^6 cells was added to the responder Clone-4 TCR splenocytes and cultured for 6 days at 37°C in a humidified incubator with 5% v/v CO_2. Clone-4 TCR transgenic effector CTL were prepared as follows: 2×10^6 splenocytes from Clone-4 TCR transgenic mice were seeded into 24-well tissue culture plates containing 6×10^6 cells/well K^{d}HA peptide-pulsed irradiated APCs and cultured for 5 days, as described above.

Cytotoxicity assay

Cells to be used as targets were prepared by incubating at 37°C with 200 μCi of sodium ^{51}chromate for 1 h in the presence or absence of various concentrations of K^{d}HA peptide, as indicated, or 1200 HA units of influenza virus A/PR/8. Cells were incubated with Vacc-WT or recombinant vaccinia viruses Vacc-HA or Vacc-K^{d}HA at an MOI of 10 plaque-forming units/cell. Target cells were washed three times, resuspended in complete RPMI, and seeded into 96-well plates at 1×10^4 cells/well in 100 μl of complete RPMI. Effector CTL were harvested and washed three times in complete RPMI and seeded into duplicate wells containing the appropriate target cells at various E:T cell ratios, making a final volume of 200 μl. Plates were incubated at 37°C in a humidified incubator with 5% v/v CO_2 for 6 h. Plates were centrifuged, and 100 μl of supernatant was removed from each well to assess isotope release using a γ-irradiation counter. The percent specific lysis was determined by the formula: percent specific lysis = (sample release − spontaneous release/maximum release − spontaneous release) × 100. All cytolytic analyses described in this work were performed at least three times.

Inoculation and growth of Renca tumor cells

Mice were inoculated s.c. into the left shoulder with 1×10^6 tumor cells in 100 μl of PBS. The size of tumors was determined using the following formula ($a^2 \times b/2$), in which a = horizontal diameter, and b = vertical diameter of the tumor mass, as determined using calipers. Tumor cell suspensions from solid tumors to be used as target cells for recognition by K^{d}HA-specific CTL were prepared as follows: mice were killed and the tumor was excised and cut into small pieces approximately 1 to 2 mm^3. These were placed into 5 ml of RPMI medium containing only 2.5 U/ml of collagenase A (Boehringer Mannheim Corp., La Jolla, CA) and agitated at 37°C for 45 min. The supernatant containing dispersed tumor cells was removed and the cells were washed three times in complete RPMI before culturing in complete RPMI.

Immunohistochemistry

Pancreata were excised and one-half was embedded in O.C.T. compound (Miles, Elkhart, IN) and frozen at −70°C. The other half was fixed in 10% v/v Formalin solution (Sigma Chemical Co.) and processed for paraffin embedding. Frozen tissues were cut using a cryomicrotome, and paraffin-embedded tissue was cut using a regular microtome. All sections were placed onto saline-coated Superfrost slides for processing (Fisher Scientific, Pittsburgh, PA). Frozen sections were fixed for 20 min in 1% w/v paraformaldehyde (Sigma Chemical Co.) in PBS, and washed for 5 min in PBS. Nonspecific binding sites were blocked using an avidin-biotin blocking kit (Vector Labs, Burlingame, CA). Sections were incubated for 1 h with affinity-purified rat Abs against mouse CD8 (PharMingen, La Jolla, CA). After washing for 10 min with PBS, sections were incubated with secondary biotinylated affinity-purified F(ab′)$_2$ mouse anti-rat IgG Abs for 1 h, and then detected using streptavidin-conjugated horseradish peroxidase (Jackson ImmunoResearch Laboratories), together with diaminobenzidine (DAB) chromagen. Paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Nonspecific binding sites were blocked by incubating with 10% v/v goat serum in PBS. Sections were incubated for 1 h with guinea pig Abs against mouse insulin (Dako Corp., Carpenteria, CA). After washing for 10 min in PBS, sections were incubated with secondary biotinylated F(ab′)$_2$ goat anti-guinea pig IgG (Vector Labs), and detected as described above. Separate serial sections of paraffin-embedded tissue were also stained with eosin (Sigma Chemical Co.), and all slides were counterstained with Mayer's hematoxylin (Sigma Chemical Co.).

Results

Growth of Renca-HA tumor cells in Ins-HA mice

Previous studies from our laboratory demonstrated that transgenic mice expressing the influenza virus HA under the control of the rat insulin promoter (Ins-HA mice) were tolerant of HA expressed by the pancreatic β cells, and did not develop autoimmune diabetes, even after immunization with influenza A/PR/8/34 (PR8) (37). However, the presence of K^{d}HA-specific CD8$^+$ T cells, obtained from either influenza virus-primed nontransgenic mice, or from Clone-4 TCR transgenic mice that express a TCR specific for a K^d-restricted peptide epitope of HA, results in autoimmune destruction of the pancreatic islet β cells in Ins-HA mice and the onset of diabetes (37, 38). To address the effect of self-tolerance upon the recognition of tumor cells expressing self epitope, the BALB/c renal carcinoma cell line (Renca) was transfected with the HA gene from PR8 (Renca-HA). Expression of the HA gene was confirmed by the presence of HA protein on the cell surface, as determined by FACS analysis using anti-HA Abs (Fig. 1*A*). Lysis of Renca-HA cells was observed using CTL obtained from Clone-4 TCR transgenic mice (38) (Fig. 1*B*). CD8$^+$ T cells obtained from Clone-4 TCR mice have specificity for the dominant

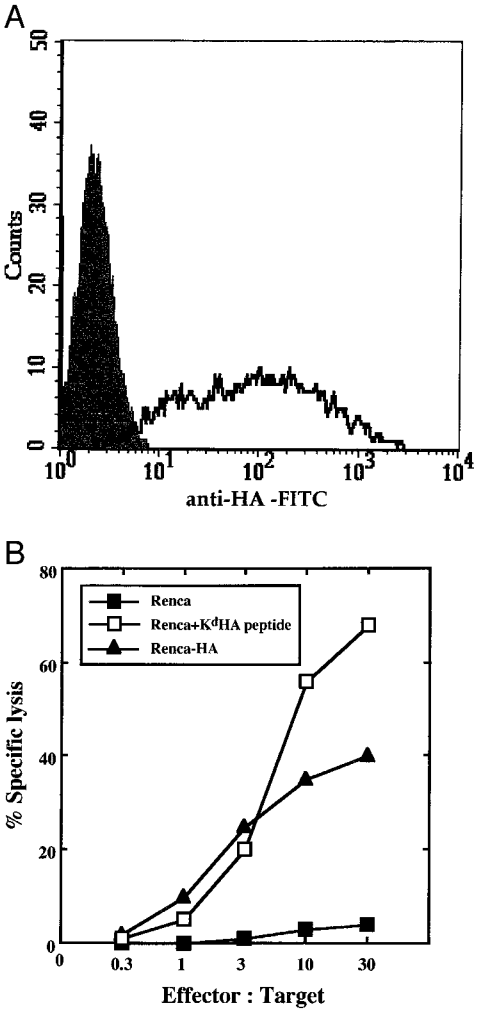

FIGURE 1. *A*, Expression of HA by Renca tumor cells. Renca (shaded area) and Renca-HA (—) tumor cells were stained with the mouse anti-HA Ab 37/38, followed by a secondary FITC-conjugated goat anti-mouse Ab. *B*, Lysis of Renca-HA tumor cells by Clone-4 TCR-bearing, K^{d}HA-specific CTL. Splenocytes from transgenic Clone-4 TCR mice were cultured with homologous, irradiated APCs pulsed with 5 μg/ml of K^{d}HA peptide. On day 5, cells were used as effectors for comparative lysis of Renca (■), Renca + K^{d}HA peptide (□), and Renca-HA (▲) tumor cells.

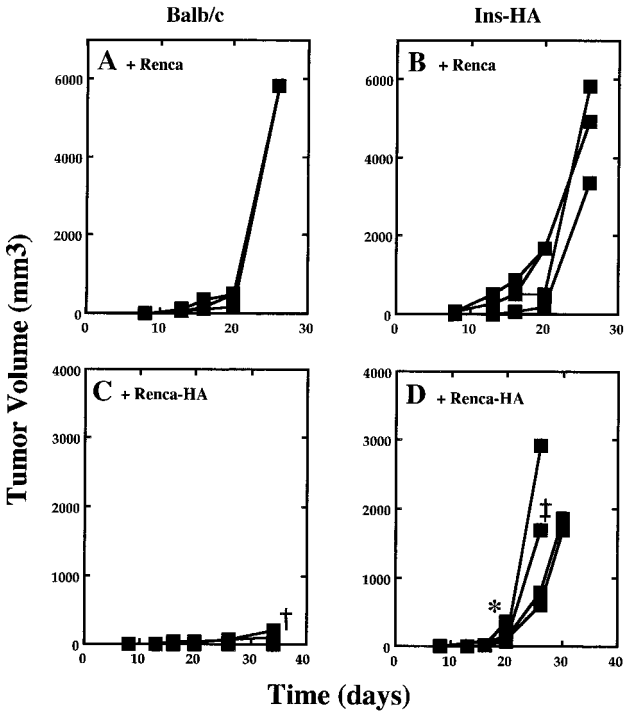

FIGURE 2. Renca-HA tumor cells grow in transgenic Ins-HA mice, but not in conventional BALB/c mice. BALB/c (*A* and *C*) and Ins-HA (*B* and *D*) mice were injected s.c. with 1×10^6 Renca-WT (*A* and *B*) or Renca-HA (*C* and *D*) tumor cells and monitored for tumor growth. Control groups (*A*, *B*, and *C*) utilized three mice per group, and experimental group (*D*) utilized six mice. *Animal was sacrificed and tumor was examined to assess maintenance of HA expression. Expression was stable, as demonstrated by recognition and lysis by HA-specific CTL. †Animal was sacrificed and tumor was examined to assess maintenance of HA expression. Expression was lost, as demonstrated by the absence of recognition and lysis by HA-specific CTL. ‡Animal was sacrificed and used as described in Figure 7.

epitope of the HA molecule, the IYSTVASSL peptide, that is presented in association with H-2K^d (40).

To determine whether expression of HA epitopes by Renca cells altered the ability of the tumor cells to grow in BALB/c or Ins-HA mice, both types of mice were compared with respect to their ability to support the growth of Renca-HA tumor cells. Injection of the parental Renca tumor cells resulted in the appearance of solid tumors in both Ins-HA mice and BALB/c mice after 8 days, and the tumors continued to grow at about the same rate in both groups of mice (Fig. 2, *A* and *B*). Renca-HA cells did not grow in conventional BALB/c mice (Fig. 2*C*), but did grow in Ins-HA mice (Fig. 2*D*). On day 35, a small tumor appeared in two of the BALB/c recipients. However, target cells prepared from the excised tumor were not lysed by K^{d}HA-specific Clone-4 CTL, indicating that these tumor cells had lost expression of HA (data not shown). In contrast, target cells prepared from tumor excised from Ins-HA recipients on day 17 following transfer were lysed by Clone-4 CTL (data not shown). These results suggested that HA expression by the tumor resulted in generation of an immune response capable of eliminating Renca HA cells in the BALB/c mouse, but not in the Ins-HA mouse.

Prior immunization with influenza virus A/PR/8 prevents growth of Renca-HA in Ins-HA mice

Recent studies in which tumor-associated self Ags have been used as a basis for tumor vaccines suggest that it may be possible to obtain effective tumor-specific immunity while maintaining self-tolerance (5, 33, 36). To determine whether the HA molecule could function as a tumor Ag in tolerant mice, we compared the growth of the Renca-HA tumor cells in unprimed Ins-HA mice and mice previously immunized with the influenza virus. Three weeks before the introduction of Renca-HA tumor cells, Ins-HA mice were primed with PR8 virus. Fifteen days after injection of the Renca-HA cells, solid tumors appeared in the unprimed Ins-HA mice (Fig. 3*A*); however, none of the Ins-HA mice immunized with PR8 developed tumors (Fig. 3*B*). Significantly, all mice remained euglycemic throughout the experiment (data not shown). However, the fact that none of these mice was diabetic did not rule out the possibility that as a consequence of tumor rejection, some degree of β cell destruction may have occurred. Thus, a histologic examination of pancreata obtained from these mice was performed. Sections of pancreatic tissue taken from unprimed Ins-HA mice showed no signs of any lymphocytic infiltration of the islets

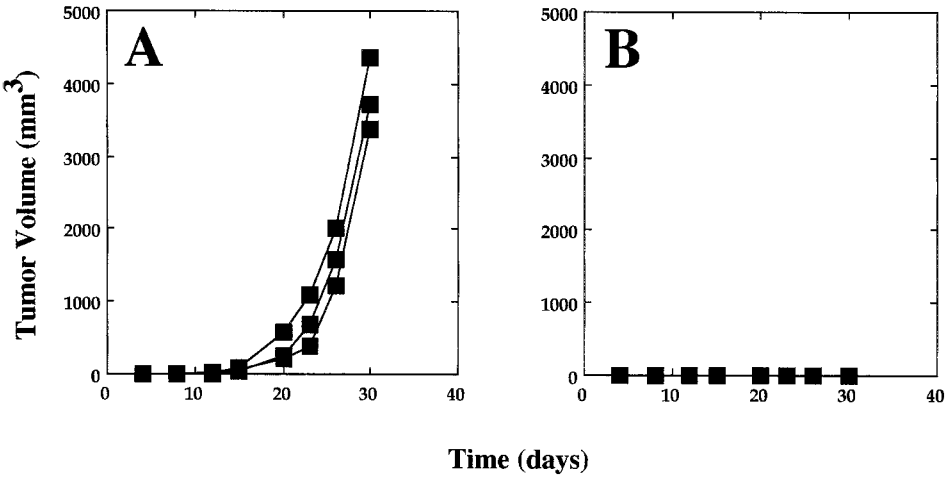

FIGURE 3. Prior immunization of Ins-HA mice with influenza virus A/PR/8 prevents growth of Renca-HA tumor cells. Ins-HA mice were injected i.p. with PBS (*A*) or influenza virus A/PR/8 (*B*) 21 days prior to s.c. injection of 1×10^6 Renca-HA tumor cells. Mice were monitored for tumor growth. Data show results for three mice in each group.

or surrounding parenchyma (Fig. 4, *A* and *B*). Islet cell clusters appeared intact and expressed high levels of insulin (Fig. 4*C*). Following immunization of Ins-HA mice with PR8, the vast majority of pancratic islets remained free of any cellular infiltration, although an extremely small number, fewer than 10%, demonstrated a peripheral localization of $CD8^+$ T cells. However, such peri-insulitis was not associated with β cell destruction, as analyses of serial sections of pancreatic tissue demonstrated uniform insulin expression by these islets. Rejection of Renca-HA cells by PR8-primed Ins-HA mice did not result in an increase in either the total number of islets demonstrating peri-insulitis, or the amount of cellularity exibited by such islets. Sections of islets that exibit the most cellularity within the pancreatic tissue are demonstrated (Fig. 4*D–F*). $CD8^+$ T cells were present at the periphery of the islets (Fig. 4*D*, arrows); however, examination of insulin expression, in serial sections of infiltrated islets, gave no evidence of any associated β cell destruction (Fig. 4, *E* and *F*). Together, these observations indicated that successful rejection of Renca-HA tumor cells by Ins-HA mice could occur while maintaining tolerance to β cells expressing HA.

Ins-HA mice contain CTL with low avidity for the dominant K^dHA epitope, IYSTVASSL

The demonstration of $CD8^+$ T cells within the periphery of some of the islets from PR8-primed Ins-HA mice, as well as the success in preventing growth of the Renca-HA cells, compelled us to examine more carefully the HA-specific T cell response in the tolerant animals. Conventional BALB/c mice respond to immunization with PR8 by providing H-2K^d-restricted CTL populations that, upon restimulation in vitro with PR8-infected APCs, demonstrate specificity for the dominant K^d-restricted epitope of the influenza virus NP and the HA, whereas only CTL specific for K^dNP were obtained from Ins-HA mice (Table I). Thus, as reported previously, tolerance to K^dHA is observed both in vivo and in vitro (37).

One effect tolerance can have on the immune system is to purge the repertoire of T cells that recognize self epitopes with high avidity, while sparing those with low avidity for the same epitopes (17, 19, 21, 24, 25, 27, 31, 32). The density of endogenously processed K^dHA epitope present on the PR8-infected APCs used in vitro may be insufficient to stimulate K^dHA-specific CTLp from Ins-HA mice, if they are of low avidity. However, it is possible that K^dHA-specific CTL could be obtained from Ins-HA mice by using high concentrations of K^dHA peptide epitope. To test this hypothesis, splenocytes from Ins-HA mice primed with PR8 were restimulated in vitro with APCs that had been pulsed with a high concentration of a synthetic peptide corresponding to the dominant K^dHA epitope, IYSTVASSL. Using these stimulation conditions, K^dHA-specific CTL could be generated in vitro from both conventional BALB/c and Ins-HA mice (Fig. 5). However, the CTL obtained from Ins-HA mice demonstrated much lower avidity for K^dHA peptide as compared with the BALB/c effector cells, requiring 100-fold more peptide to achieve comparable lysis. Such low avidity CTL are not specific for impurities within the peptide preparation, as evidenced by the fact that there is an absolute requirement for prior immunization of Ins-HA mice with PR8 to generate these CTL in vitro (data not shown). Furthermore, target cells infected with a minigene encoding the K^dHA peptide, IYS TVASSL (Vacc-K^dHA), were also lysed by such CTL (Fig. 6). Therefore, in Ins-HA mice, tolerance of HA was associated with a T cell repertoire devoid of high avidity K^dHA-specific CTL. Only targets presenting high densities of the K^dHA peptide are lysed by these low avidity CTL.

To determine whether the presence of Renca-HA tumor cells in vivo resulted in priming of HA-specific CTL, splenocytes from Ins-HA mice and BALB/c mice were stimulated in vitro with K^dHA peptide-pulsed or PR8-infected APCs. Figure 7 shows that following injection with Renca-HA tumor cells, BALB/c mice were able to generate CTL having high avidity for K^dHA peptide-pulsed target cells, whereas K^dHA-specific CTL could not be obtained from Ins-HA mice even after cells were cultured with APCs that had been pulsed with a high concentration of K^dHA peptide.

K^dHA-peptide-specific CTL reject Renca-HA cells in Ins-HA mice

These data demonstrated that prior immunization of Ins-HA mice with PR8 could prevent the growth of Renca-HA cells. However, as such immunization could also lead to stimulation of Ab and/or T cells with specificity for portions of the HA other than the IYS TVASSL peptide, it was of interest to determine whether the priming of CTL specific for this particular epitope was actually sufficient for tumor rejection. To this end, Ins-HA mice were infected

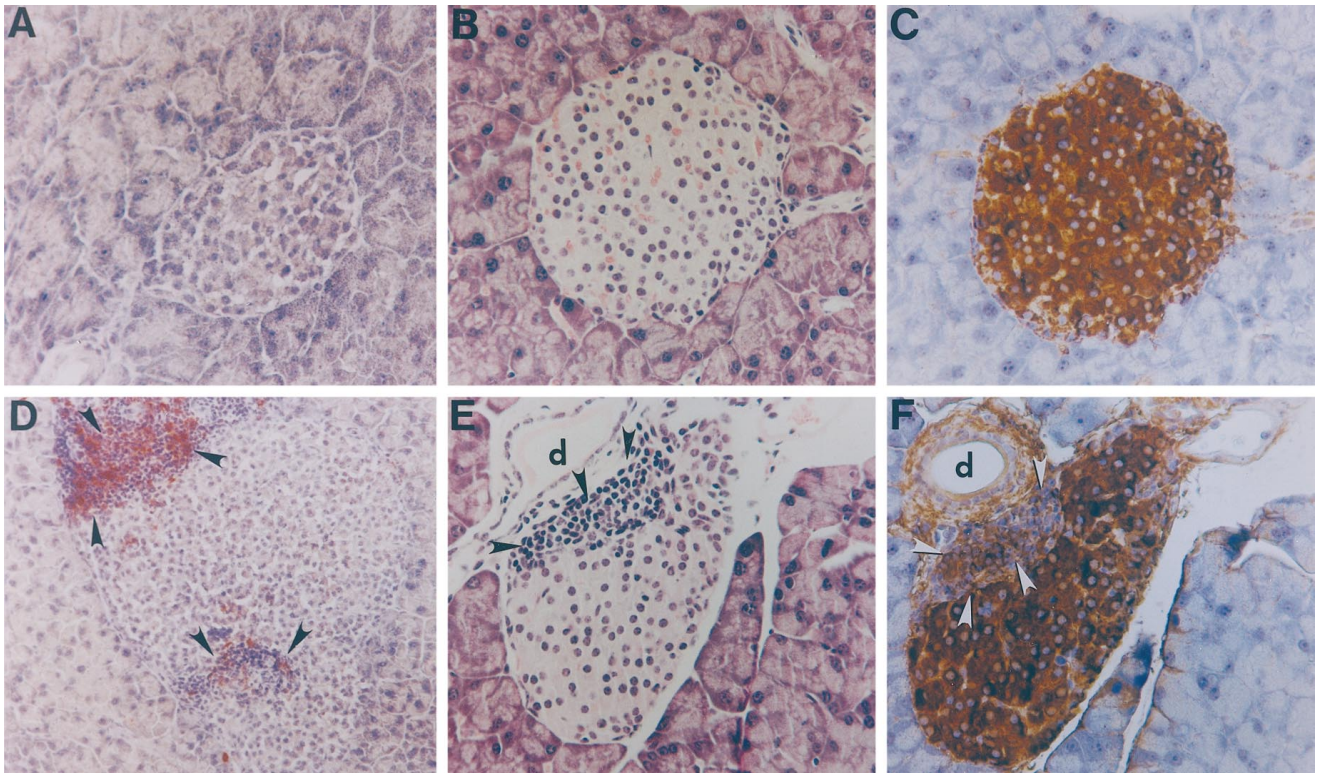

FIGURE 4. Immunohistologic analysis of pancreata from Ins-HA mice. Sections (*A–C*) are prepared from pancreatic tissue obtained from an unprimed Ins-HA mouse, and sections (*D–F*) are prepared from an influenza virus A/PR/8-primed Ins-HA mouse that had rejected Renca-HA tumor cells. *Section A* is a cryostat section of pancreatic tissue stained for CD8 expression by the indirect immunoperoxidase technique using DAB as a chromogen, and counterstained with hematoxylin (×200). $CD8^+$ T cells are not present in the pancreas of this animal. *Section B* shows paraffin-embedded pancreatic tissue isolated from the same mouse stained with hematoxylin and eosin (H+E; ×200). Islets appear free from any cellular infiltration. *Section C* is a serial section from the same paraffin-embedded pancreatic tissue stained for insulin by the immunoperoxidase technique, using DAB as a chromogen, and counterstained with hematoxylin (×200). Note the appearance of intact islet β cell clusters. *Sections D–F* are prepared from pancreatic tissue obtained from an influenza virus A/PR/8-primed Ins-HA mouse having rejected Renca-HA tumor cells. Sections of islets that exibit the most cellularity within the tissue are demonstrated. *Section D* is a cryostat section of pancreatic tissue stained for CD8 expression (×100). Note that $CD8^+$ T cells are present only at the periphery of the islets forming a peri-insulitis (arrows). *Section E* shows paraffin-embedded pancreatic tissue isolated from the same mouse stained with hematoxylin and eosin (H+E; ×200). Once again, localization of lymphocytes occurs only at the periphery of the islet (arrows), adjacent to the pancreatic duct (d). *Section F* is a serial section from the same parafin-embedded pancreatic tissue stained for insulin expression and counterstained with hematoxylin (×200). Note that peri-insulitis is not associated with any β cell destruction or disruption of the pancreatic architecture.

Table I. *Tolerance among HA-specific CTLp from transgenic Ins-HA mice*

		% Specific Lysis of B10.D2 (H-2^d) Target Cells					
Effectors	E:T	Alone	+PR8	+K^dNP	+K^dHA	+Vacc-WT	+Vacc-HA
BALB/c	10	7	47	68	50	11	49
	3	2	40	40	22	1	29
	1	1	19	12	9	0	11
Ins-HA	10	13	40	63	6	12	13
	3	4	20	32	3	6	10
	1	2	5	11	6	0	7

Ins-HA mice and conventional BALB/c mice were immunized i.p. with 1200 HA units of influenza virus A/PR/8 21 days prior to sacrifice. Spleen cells were cultured with PR8-infected, irradiated homologous APCs. On day 6 cells were assayed for the presence of HA-specific CTL by lysis of virus-infected and peptide-pulsed B10.D2 (H-2^d) target cells as indicated. Data are representative of two experiments, each utilizing two mice per group.

with Vacc-K^dHA, before receiving Renca-HA cells. As was observed following immunization with PR8, in vitro restimulation of splenocytes obtained from Vacc-K^dHA-primed Ins-HA mice with K^dHA peptide-pulsed APCs resulted in a population of CTL that demonstrated low avidity for K^dHA (Fig. 5). As described in Figure 8, Renca-HA cells grew in Ins-HA mice previously immunized with Vacc-WT, whereas tumor cells were rejected following transfer into Ins-HA mice that received the Vacc-K^dHA. Thus, vaccination of Ins-HA mice with the dominant K^dHA peptide was sufficient to prevent the growth of Renca-HA tumor cells.

Discussion

Recent studies have revealed the potential for self epitopes to serve as targets for immunotherapy. In order for this approach to be successful, it is necessary to mobilize a T cell repertoire that is normally tolerant of these same epitopes. However, the degree of T cell tolerance imposed by individual self epitopes can differ significantly. The experiments described in this work demonstrate the potential to prevent growth of tumor cells expressing a self epitope that exists in the immune repertoire after successful self-tolerance. Although not all of the mechanisms responsible for tolerance to the HA transgene in Ins-HA mice have yet been defined, it is clear that the Ins-HA transgene is an extremely effective toleragen, as even

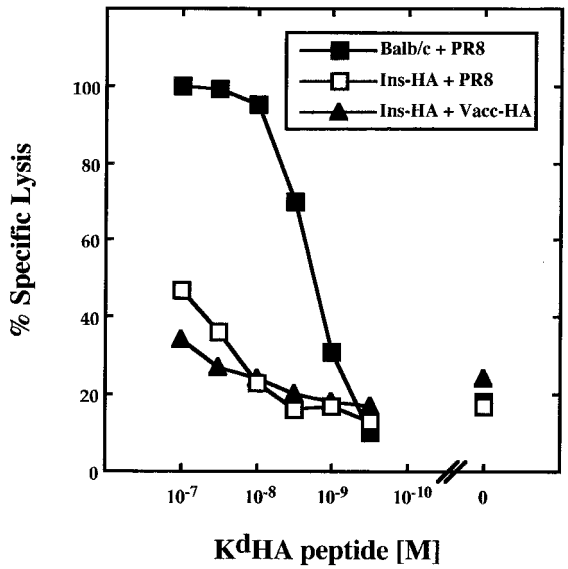

FIGURE 5. Retrieval of K^dHA-specific CTL from transgenic Ins-HA mice primed with influenza virus A/PR/8. BALB/c mice (■) and Ins-HA mice were immunized with influenza virus A/PR/8 (□) and recombinant vaccinia virus Vacc-K^dHA (▲) 21 days prior to sacrifice. Splenocytes were removed and cultured with homologous irradiated APCs pulsed with 5 μg/ml of K^dHA peptide. On day 6, cells were assayed for the presence of K^dHA-specific CTL by lysis of ^{51}Cr-labeled B10. D2 target cells pulsed with the indicated concentration of K^dHA peptide. The E:T was 30:1. Data are representative of at least two separate experiments, each utilizing two mice per group.

after immunization with either the influenza virus A/PR/8 or recombinant vaccinia virus Vacc-K^dHA, tolerance to β cell-expressed HA was maintained. Furthermore, stimulation of splenocytes obtained from PR8-infected Ins-HA mice, with PR8-infected APCs, did not yield HA-specific CTL. Nevertheless, in vitro stimulation with high concentrations of a K^dHA peptide revealed the presence of residual, low avidity CTL with specificity for a dominant K^d-restricted HA peptide epitope. Apparently, such CTL have escaped by virtue of their low avidity for this self epitope. Similar observations concerning persistence of low avidity T cells in the presence of a toleragenic self protein have been described by several investigators (17, 21, 24, 25, 27, 32).

We first compared conventional BALB/c mice and Ins-HA mice with respect to their ability to support the growth of tumor cells that express HA. Although the untransfected parental cells (Renca) grew progressively in both mouse lines, Renca-HA cells were rejected by BALB/c mice. This, together with the fact that K^dHA-specific CTL were detectable in BALB/c mice, indicated that the anti-HA immune response in BALB/c mice is successful in eliminating tumor cells expressing a high concentration of this foreign protein. It is not yet clear as to the identity of the APCs involved in presentation of Ag to K^dHA-specific CTLp. Studies in other tumor models have concluded that tumor cells generally do not directly stimulate T cells, and that the Ags must first be processed by professional APCs that are better able to provide both Ag and costimulatory molecules required for T cell stimulation (41, 42). HA expression by the tumor may provide abundant Ag for APC processing through the cross-priming pathway (43, 44). However, the dependence of T cells on costimulation is inversely related to the amount of Ag available for stimulation (45, 46). Renca-HA cells do not express the B7 costimulatory molecule (data not shown), thus it would be anticipated that only those cells with relatively high avidity for HA epitopes could be activated in the absence of appropriate costimulatory signals (38, 45, 47–51). In conventional BALB/c mice, it is possible that Renca-HA cells may themselves directly stimulate high avidity CTLp. Considering that HA is expressed at relatively high levels by these cells, it would be of interest to determine whether the HA-specific immune response in BALB/c mice is as effective in a situation in which the Ag density is somewhat lower. Future experiments will address this issue.

Importantly, Ins-HA mice that had received the Renca-HA cells demonstrated progressive growth of the tumor and did not demonstrate K^dHA-specific CTL. The failure of tumor cells to activate residual low avidity K^dHA-specific T cells present in Ins-HA mice is consistent with a requirement for high epitope density as well as costimulation to stimulate these cells. These requirements were fulfilled by the use of influenza PR8 or Vacc-K^dHA as the immunogen. Priming the Ins-HA mice with either of these viruses resulted in a population of memory cells that was able to subsequently eliminate the Renca-HA cells. It has been proposed that tolerance to self epitopes most affects those T cells that are specific for dominant epitopes, and may spare T cells with specificity for cryptic epitopes from these same proteins (14, 52). The fact that recognition of the dominant K^d-restricted HA epitope presented by Vacc-K^dHA was sufficient for tumor rejection, indicates that low avidity cells, specific for dominant epitopes, can be recruited for tumor elimination. The priming of CTL specific for this K^dHA epitope may have been facilitated by the generation of a helper response to vaccinia Ags, bypassing tolerance of HA-specific $CD4^+$ T cells that we previously demonstrated in Ins-HA mice (37). We have ruled out a need for the participation of HA-specific Abs in the tumor rejection process as, following immunization of Ins-HA mice with PR8, HA-specific Abs were readily observed in the serum of the Ins-HA mice; none was detected in sera from mice that had been immunized with Vacc-K^dHA (data not shown).

Our results present the rather paradoxical findings that, although tolerance to a self epitope presented by the pancreas can be maintained, the immune response to this same epitope can succeed in preventing growth of tumor cell expressing the same Ag. Several factors may contribute to this observation. First, it has been demonstrated that T cell avidity can determine the success of in vivo T cell function. For example, low avidity CTL are unable to function in viral clearance in vivo (53, 54). K^dHA-specific CTL obtained from Ins-HA mice represent such low avidity T cells, and accordingly, the inability of these primed T cells to cause diabetes is most likely due to their low avidity, rather then their access to the pancreas. Indeed, histologic examination revealed that although $CD8^+$ T cells are present at the periphery of the islets, they remained intact. However, it still remains to be explained how these same T cells succeed in tumor elimination. One factor that cannot be dismissed easily is that trauma induced by the tumor injection may be accompanied by a degree of inflammation that facilitates rejection by a primed T cell response. Perhaps the simplest explanation would be that there is a higher density of K^dHA epitope presented on the transfected Renca-HA cells than on the islet β cell. Thus, it would be predicted that tumor cells expressing less K^dHA epitope may not be eliminated in Ins-HA mice preimmunized with influenza. Alternatively, other factors may exist that protect the β cells by dampening the activation status of the T cells.

This study demonstrates the successful use of a vaccine vector expressing a self epitope that is capable of eliciting an immune response against tumor cells expressing the same self epitope; yet

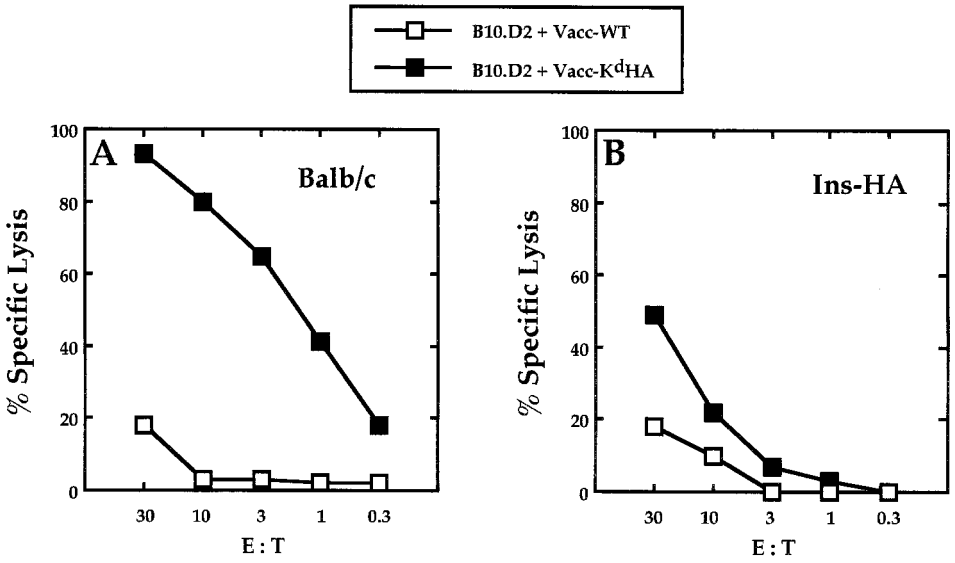

FIGURE 6. K^dHA peptide-specific CTL also recognize Vacc-KdHA virus-infected target cells. Splenocytes from PR8-infected BALB/c (*A*) and Ins-HA (*B*) mice were cultured with homologous irradiated APCs pulsed with 5 μg/ml of K^dHA peptide. On day 5, cells were used as effectors for comparative lysis of B10. D2 target cells infected with Vacc-WT (□) and Vacc-K^dHA (■), as indicated. Data are representative of at least two separate experiments, each utilizing two mice per group.

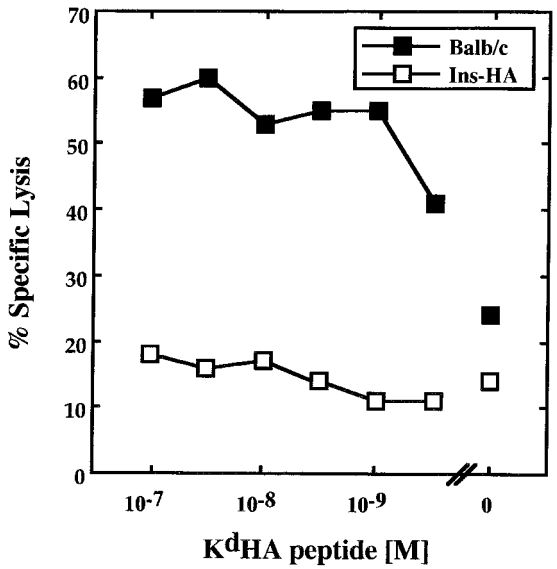

FIGURE 7. Renca-HA tumor cells induce K^dHA-specific CTL in conventional BALB/c mice, but not transgenic Ins-HA mice. BALB/c (■) and Ins-HA (□) mice were sacrificed 24 days following s.c. injection with 1×10^6 Renca-HA tumor cells. Splenocytes from each group of mice were cultured with homologous, irradiated APCs infected with PR8, or pulsed with 5 μg/ml of K^dHA peptide, respectively. On day 6, cells were assayed for the presence of K^dHA-specific CTL by lysis of ^{51}Cr-labeled B10. D2 target cells pulsed with the indicated concentrations of K^dHA peptide. Data are representative of two separate experiments, each utilizing two mice per group.

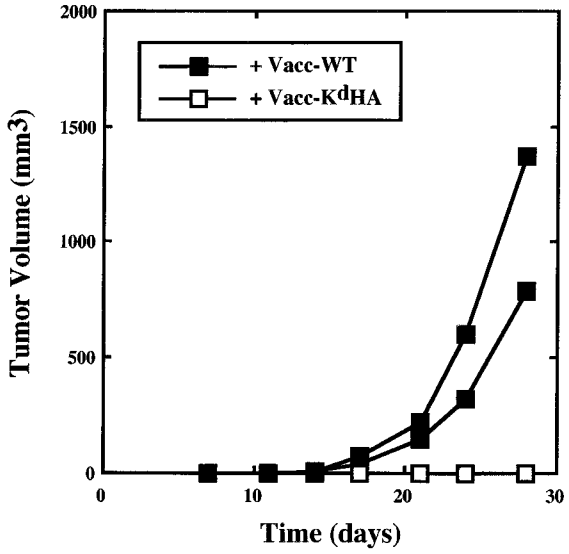

FIGURE 8. Preimmunization of Ins-HA mice with Vacc-K^dHA results in rejection of Renca-HA tumor cells. Ins-HA mice were injected i.p. with either Vacc-WT (■) or recombinant vaccinia virus (Vacc-K^dHA; □) 21 days prior to s.c. injection of 1×10^6 Renca-HA tumor cells. Mice were monitored for tumor cell growth. Control group immunized with Vacc-WT utilized two mice, and experimental group immunized with Vacc-K^dHA utilized three mice.

at the same time, tolerance to peripherally expressed HA is maintained. This model is analogous to expression of a variety of naturally occurring Ags that are up-regulated in their expression in tumor cells. Indeed, recent findings from several laboratories have demonstrated the success of vaccination against p53 and its antigenic peptides in preventing growth of tumors that express high levels of this tumor-associated protein (33, 36). Although low level expression of p53 is known to occur in a variety of tissues (55–57), no experiments were conducted that determined whether or not self-tolerance had affected the T cell repertoire available for recognition of epitopes from p53 expressed by the tumor cells. Recent studies comparing responses to epitopes of p53 in p53-deficient and p53-sufficient mice have demonstrated a reduction in the response to A2-restricted epitopes that is attributable to self-tolerance (58). Thus, it is likely that some degree of tolerance to p53 normally occurs, and that immunization succeeded in mobilizing the residual repertoire.

There are a number of major concerns regarding the immunization of individuals with vaccines containing self epitopes. If tolerance to these epitopes is broken, then populations of CTL may be generated that not only demonstrate tumor-specific cytotoxicity, but also have the capability of engaging in autoimmune destruction of other tissues expressing the self epitope. Studies with the Ins-HA model may help to further define the limitations of this type of tumor therapy, and also elucidate the most successful forms of tumor immunotherapy.

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