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Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction

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ABSTRACT **Vaccination with synthetic peptides representing cytotoxic T lymphocyte (CTL) epitopes can lead to a protective CTL-mediated immunity against tumors or viruses. We now report that vaccination with a CTL epitope derived from the human adenovirus type 5 E1A-region ($Ad5E1A_{234-243}$), which can serve as a target for tumor-eradicating CTL, enhances rather than inhibits the growth of Ad5E1A-expressing tumors. This adverse effect of peptide vaccination was rapidly evoked, required low doses of peptide (10 μg), and was achieved by a mode of peptide delivery that induces protective T-cell-mediated immunity in other models. Ad5E1A-specific CTL activity could no longer be isolated from mice after injection of Ad5E1A–peptide, indicating that tolerization of Ad5E1A-specific CTL activity causes the enhanced tumor outgrowth. In contrast to peptide vaccination, immunization with adenovirus, expressing Ad5E1A, induced Ad5E1A-specific immunity and prevented the outgrowth of Ad5E1A-expressing tumors. These results show that immunization with synthetic peptides can lead to the elimination of anti-tumor CTL responses. These findings are important for the design of safe peptide-based vaccines against tumors, allogeneic organ transplants, and T-cell-mediated autoimmune diseases.**

Cytotoxic T lymphocytes (CTL) can play an important role in the defense against experimental and human malignancies (1, 2). CTL recognize small antigenic peptide fragments in the context of class I major histocompatibility molecules (3). These peptides are mostly generated from endogenously synthesized proteins.

Vaccination with synthetic peptides corresponding to CTL epitopes can induce protective CTL-mediated immunity in a variety of model systems (4). For instance, immunization of mice with synthetic peptides deduced from proteins of either Sendai virus or lymphocytic choriomeningitis virus (LCMV) led to the induction of protective immunity against a subsequent challenge of respectively Sendai virus or LCMV (5, 6). In tumor models, protective immunity was established by immunization with tumor-specific synthetic peptides. Immunization with a peptide derived from the human papillomavirus type 16 (HPV16) led to the protection against a lethal dose of HPV16-transformed tumor cells (7, 8), and vaccination with a peptide encompassing a CTL epitope derived from chicken ovalbumin led to the induction of protective immunity against a thymoma transfected with the cDNA of chicken ovalbumin (9). The approach of using synthetic peptide CTL epitopes for the induction of CTL responses is now being applied in human beings. Peptides encoded by the HPV16 early region 6 and 7 (E6 and 7) oncogenes that are immunogenic to human CTL have been identified (10) and are currently employed at our hospital in a phase I/II peptide vaccination study. Vaccination of healthy volunteers with the hepatitis B core antigen peptide 18–27 linked to a T-helper epitope and two palmitic acid molecules induced a hepatitis B core antigen peptide-specific CTL response cross-reactive on virus-infected cells (11).

While these results provide the basis for the development of peptide-based prophylactic and therapeutic anti-tumor vaccines, vaccination with synthetic peptides may also lead to T cell unresponsiveness (12, 13). At least three repetitive intraperitoneal (i.p.) immunizations with a relatively high dose (100 μg/mouse) of synthetic peptide deduced from the glycoprotein of LCMV induced specific T-cell tolerance, and this mode of peptide delivery prevented the induction of diabetes by infection with LCMV in a transgenic mouse model in which LCMV glycoprotein was expressed in the β islet cells of the pancreas (12). Apparently, there is a balance between induction of T-cell responses and tolerization by injection of antigenic peptides. These differences in outcome of peptide vaccination could have a strong impact on the design, delivery, and development of peptide-based anti-tumor vaccines, but will also have implications for vaccination strategies against T-cell-mediated autoimmune diseases and other harmful T-cell-mediated immune destructions. In this paper we show that a single vaccination with a low dose of a subcutaneously (s.c.) given peptide comprising a CTL-epitope derived from the human adenovirus type 5 early region 1A (Ad5E1A) oncogene promotes rather than suppresses the outgrowth of Ad5E1A-expressing tumor cells in normal immunocompetent mice. The inhibitory effects of peptide vaccination were also noted on adoptively transferred Ad5E1A-specific CTL clones and in T-cell receptor (TCR) transgenic mice. The implications of these findings for the development of peptide-based intervention protocols against T-cell-mediated autoimmune diseases, allogeneic organ transplants, viral infections, and malignancies are discussed.

MATERIALS AND METHODS

Mice. C57BL/6 (B6 Kh, H-2^b) mice were obtained from the Netherlands Cancer Institute (Amsterdam) and C57BL/6 *nu/nu* (B6 nude) were obtained from Bomholtgard (Ry, Denmark). The TCR transgenic mice express the TCR-α and -β chains derived from the H-$2D^b$-restricted, $Ad5E1A_{234-243}$-specific CTL clone 5 (14). This T cell uses rearranged Vα16 and Vβ1 TCR chains. The cDNAs encoding the complete α and β chains were inserted into an expression construct based on genomic sequences of the human CD2 gene (15), and the resulting transgenes were coninjected into blastocysts of C57BL/6 (B6 Kh) mice.

Cell Lines and Culture Conditions. Cells expressing Ad5E1A and EJ*ras* (AR-cell lines) were generated by transfection with pAd5E1A (*Pst*I) (16), pEJ*ras* (17), and pTK-neo

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Abbreviations: Ad5E1, adenovirus type 5 early region 1; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocyte; HPV, human papillomavirus; IFA, incomplete Freund's adjuvant; IL-2, interleukin 2; rIL-2, recombinant IL-2; LCMV, lymphocytic choriomeningitis virus; TCR, T-cell receptor.

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(14). Expression of Ad5E1A and EJ*ras* gene products was confirmed both at the mRNA and protein level (data not shown). All other cell lines used were generated as described (14, 18). All cells were maintained as described elsewhere (7, 14, 18).

Peptides. Peptides were generated by solid phase strategies on a ABIMED 422 synthesizer (ABIMED, Langenfeld, Germany) as described (19). Peptides were stored dry at −70°C. The purity of the peptides was determined by analytical phase HPLC using water-acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) and proved to be at least 85% (UV, 214 nm). The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry (TOF-MALDI) on a lasermat mass spectrometer (Finnigan–MAT, Herts, UK). About 5 pmol of the peptide in 0.5 μl water/acetonitrile containing 0.1% TFA was mixed with 0.5 μl of matrix solution [ACH; 10 mg/ml in acetonitrile/water, 60/40 (vol/vol) containing 0.1% TFA] and applied to the instrument. Calibration was performed with peptides of known molecular mass.

Peptide Immunization and Challenge with Ad5E1A + ras Transformed Tumor Cells. Peptide immunizations were performed as previously described (5–7). Peptides dissolved in 100 μl phosphate-buffered saline (PBS) were extensively mixed with 100 μl incomplete Freund's adjuvant (IFA) and 0.5% (wt/vol) of bovine serum albumin (BSA). The 200 μl mixture was s.c. injected in B6 mice. Two weeks later mice were challenged with 10^7 Ad5E1A + ras transformed tumor cells (clone AR 6) or 0.5×10^6 HPV16-transformed tumor cells (clone HPVC3) s.c. in 300 μl PBS.

Limiting Dilution. CTL clone 5 (1.5×10^7) (14) was administered intravenously in normal immunocompetent mice. Three days later 100 μg of the Ad5E1A-encoded CTL epitope $Ad5E1A_{234-243}$ (sequence, Ser-Gly-Pro-Ser-Asn-Thr-Pro-Pro-Glu-Ile) or the $Ad5E1B_{192-200}$-encoded (control) CTL epitope (sequence, Val-Asn-Ile-Arg-Asn-Ile-Cys-Cys-Tyr-Ile) (18) in PBS was mixed with IFA plus 0.5% (wt/vol) BSA and s.c. injected. Six days later spleen cells of these animals were plated out in a limiting dilution assay. Twenty-four replicate microcultures were set up per serial dilution of responder spleen cells and 5×10^3 irradiated (25 Gy) interferon-γ (2 days, 10 units/ml) Ad5E1-transformed cells in U-bottomed tissue culture plates. After 7 days of culture in the presence of 10 Cetus Units of recombinant interleukin 2 (rIL-2) the microcultures were split into three and tested in an Europium- (Eu^{3+}) release assay (7) on 10^3 Eu^{3+}-labeled syngeneic target cells in a total volume of 100 μl in the presence of 0.5 μM of peptide $Ad5E1A_{234-243}$ (14), peptide $Ad5E1B_{192-200}$ (18), or the HPV16 $E7_{49-57}$-encoded peptide Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe (7). Microcultures were scored as responding and nonresponding cultures. Responding cultures were defined as those in which the Eu^{3+} release value exceeded the mean background Eu^{3+} release plus three times the standard deviation. Limiting dilution analysis predicts that if 37% of the tested microcultures is negative, then for that given responder cell concentration there is an average of one CTL-precursor per well (20). CTL-precursor frequencies were only taken into account if the goodness of fit was ≤12.5 (jacknife method; ref. 21).

***In Vivo* Administration of Tumor-Specific CTL Clones.** *In vivo* therapy for Ad5E1-induced tumors with tumor-specific CTL clones was performed as described (14). In short, B6 *nu/nu* mice with Ad5E1-induced tumors ranging from 40–50 mm^3 were treated with intravenous injections of B6 Ad5E1A-specific CTL clone 5 (1.5×10^7) in combination with 10^5 Cetus Units rIL-2, administered s.c. mixed with 100 μg of peptide $Ad5E1A_{234-243}$ or 100 μg of peptide $Ad5E1B_{192-200}$ as a control in IFA containing 0.5% BSA at a site distant from the tumor. The mixtures of peptide/IL-2 and IFA were prepared as described above.

RESULTS

Generation of Ad5E1A + ras Transformed Tumor Cells. Previously, we described a CTL epitope encoded by the Ad5E1A region ($Ad5E1A_{234-243}$). This CTL epitope is presented to the immune system in the context of H-$2D^b$, and CTL clones directed against this epitope are able to eradicate large established Ad5E1-induced tumors in B6 nude mice (14, 22). This indicates that the Ad5E1A-encoded epitope is able to elicit CTL capable of mediating tumor regression of Ad5E1A-expressing tumors *in vivo*. To test whether vaccination with this CTL epitope is also able to induce protective immunity against Ad5E1A-expressing tumor cells in immunocompetent mice, we generated tumor cells transformed by the Ad5E1A region and an activated *ras* oncogene. The Ad5E1A + ras transformed cells are recognized by Ad5E1A-specific CTL clone 5, showing that these tumor cells present the $Ad5E1A_{234-243}$-encoded CTL epitope (data not shown). Moreover, Ad5E1A + ras transformed tumor cells, in contrast to Ad5E1-transformed cells, are tumorigenic in immunocompetent mice, so that they can be used to study the effects of vaccination with peptide $Ad5E1A_{234-243}$ on the induction of protective CTL mediated immunity against tumor outgrowth *in vivo*. A dose of 10^7 tumor cells injected s.c. causes growth of small tumors in 80–100% of the mice. By day 40, ≈10–30% of the animals die because of a progressively growing tumor. The other animals, which have developed a tumor, still carry it or have eradicated the tumor (5–10% of the animals) (data not shown), indicating that Ad5E1A + ras transformed tumor cells are weakly immunogenic in immunocompetent mice.

Immunization with the Ad5E1A-Encoded CTL Epitope Leads to an Enhanced Outgrowth of Ad5E1A + ras Expressing Tumor Cells. Immunocompetent B6 mice were immunized once s.c. with peptide $Ad5E1A_{234-243}$ in IFA, a vaccination protocol that has been successfully used to induce protective immunity in several other models (5–7). Two weeks later the mice were challenged with Ad5E1A + ras transformed tumor cells. Control mice immunized with irradiated tumor cells were protected against the outgrowth of Ad5E1A + ras transformed tumor cells, indicating that protective immunity can be established against these tumor cells (Fig. 1). Unexpectedly, mice immunized with the Ad5E1A peptide were not protected against the outgrowth of these tumor cells. Instead, the tumors in these animals grew faster than the tumors in animals that were injected with IFA only, or with the H-$2D^b$-binding control peptide HPV16 $E7_{49-57}$ in IFA (Fig. 1*A*). All animals vaccinated with the Ad5E1A peptide died within 40 days after tumor challenge, whereas at that time almost all animals in the control groups were still alive (Fig. 1). These results indicate that immunization with peptide $Ad5E1A_{234-243}$ leads to the inability of immunized mice to control the outgrowth of Ad5E1A-expressing tumors. This effect is induced very rapidly, since injection of 10 μg of the Ad5E1A peptide at the left flank and a tumor cell challenge on the right flank of the animal on the same day resulted in the enhancement of tumor outgrowth (Fig. 1*C*).

To define the minimum amount of peptide required to observe the enhancement of tumor outgrowth, we titrated the injected dose of peptide (Fig. 2). Mice injected with 10 μg of the Ad5E1A peptide develop rapidly growing tumors earlier and died sooner than animals injected with lower doses of peptide or an H-$2D^b$-binding control peptide ($P = 0.004$; log-rank test) (Fig. 2). Protective immunity against the outgrowth of Ad5E1A + ras cells could not be induced at any concentration of peptide (ranging from 10 μg to 1 ng peptide per mouse), since the tumor-take in mice receiving the Ad5E1A peptide was the same as the tumor-take in mice receiving the Ad5E1B control peptide (data not shown).

Administration of the Ad5E1A peptide, and not of other peptides (Figs. 1 and 2), leads to the enhanced outgrowth of

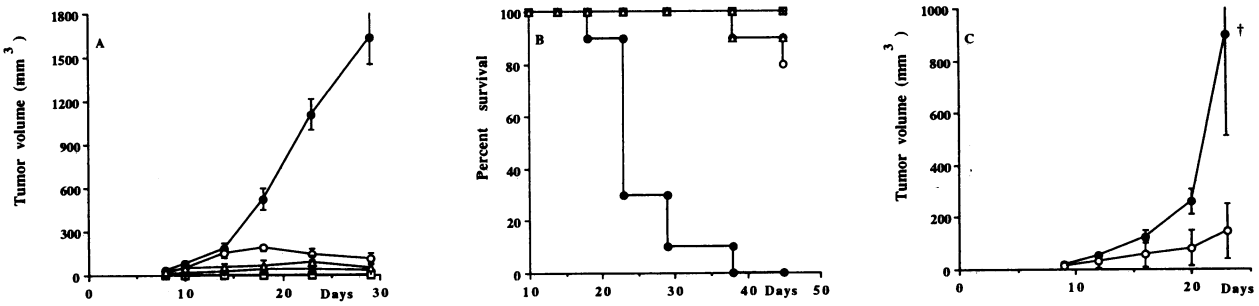

FIG. 1. Vaccination with the Ad5E1A peptide results rapidly in the inability to control the outgrowth of Ad5E1A + ras transformed tumor cells. Mice were immunized s.c. with irradiated Ad5E1A + ras cells in PBS (□), left untreated (—), immunized with 50 μg of peptide Ad5E1A$_{234-243}$ in IFA (●), with 50 μg control peptide HPV16 E7$_{49-57}$ in IFA (○), or with IFA only (△). Two weeks later the mice were challenged with live Ad5E1A + ras cells on the other flank. Tumors in mice immunized with Ad5E1A peptide grow more rapidly compared with tumors in animals treated with control peptide ($P < 0.0001$; one-sided Student's t test). Mean tumor volumes ± SEM ($n = 10$) are shown in mm^3 (A). Mice immunized with Ad5E1A peptide die sooner because of a progressively growing Ad5E1A + ras tumor compared with mice injected with control peptide ($P = 0.0006$; log-rank test) (B). The enhanced outgrowth of Ad5E1A + ras transformed tumor cells is induced rapidly after peptide vaccination (C). Mice were immunized s.c. on day zero with 10 μg of the Ad5E1A peptide in IFA or with 10 μg of the HPV16 E7-encoded control peptide in IFA and on the same day, on the other flank, injected with live Ad5E1A + ras transformed tumor cells. Mice receiving Ad5E1A peptide develop bigger tumors than mice injected with control peptide. Mean tumor volumes ± SEM ($n = 5$) are shown in mm^3.

Ad5E1A-expressing tumors. This shows that these tumors do not grow more rapidly when control peptides are injected, but only when the Ad5E1A peptide is given. Thus, this effect is peptide-specific. To show that immunization with the Ad5E1A peptide leads to the enhanced outgrowth of Ad5E1A-expressing tumors, and not of other tumor types, we immunized B6 mice with the HPV16 E7$_{49-57}$-derived CTL epitope mixed with the Ad5E1A$_{234-243}$ peptide. Mice were challenged with HPV16-transformed tumor cells. Immunization with peptide HPV16 E7$_{49-57}$ induces protective CTL-mediated immunity against HPV16-induced tumors (7). Mice immunized with the mixture of Ad5E1A and HPV16 E7 peptides in IFA are equally well protected against a subsequent challenge of HPV16-transformed tumor cells as mice immunized with HPV16 E7 peptide in IFA only (Fig. 3). Moreover, tumor growth rates of HPV16-transformed tumor cells in mice immunized with only Ad5E1A$_{234-243}$ was the same as the tumor growth rate in control mice (data not shown). These data indicate that injection of peptide Ad5E1A$_{234-243}$ does not result in a general inability of the mice to reject tumors. Taken together, these observations suggest that immunization with the Ad5E1A-encoded CTL epitope leads to a specific functional deletion of Ad5E1A-specific immunity, resulting in an inability to reject Ad5E1A-expressing tumors.

Ad5E1A-Specific Activity Cannot Be Detected After Administration of the Ad5E1A Peptide. Immunization with the Ad5E1A peptide leads to an enhanced outgrowth of Ad5E1A-expressing tumors. This suggests that Ad5E1A-specific CTL are tolerized by administration of the Ad5E1A-encoded CTL epitope. To study the effect of administration of the Ad5E1A peptide on Ad5E1A-specific CTL activity, Ad5E1A-specific CTL clone 5 was adoptively transferred into naive immunocompetent animals. Three days later peptide Ad5E1A$_{234-243}$, or an Ad5E1B$_{192-200}$-encoded control peptide (17) were administered. The presence of CTL clone 5 in the spleen of these animals was functionally tested 6 days later. CTL clone 5 could only be recovered from animals that received CTL clone 5 and the Ad5E1B control peptide, but not from animals that received CTL clone 5 and the Ad5E1A peptide (Table 1). Administration of the Ad5E1A peptide had no effect on the presence of adoptively transferred Ad5E1B-specific CTL clones (data not shown). To study whether the Ad5E1A peptide had also an effect on naive Ad5E1A-specific CTL, B6 mice expressing a transgenic TCR specific for the Ad5E1A peptide were injected with the Ad5E1A peptide. In contrast to splenocytes from normal B6 mice, splenocytes from naive TCR-transgenic mice, when restimulated *in vitro* with Ad5E1A peptide-loaded lipopolysaccharide blasts, display strong cytolytic activity against Ad5E1-transformed cells. After injection of the Ad5E1A peptide, but not of an irrelevant control peptide, the spleen cell cultures derived from Ad5E1A-specific TCR-transgenic animals are no longer able to lyse Ad5E1-transformed tumor cells (Fig. 4). Taken together, the com-

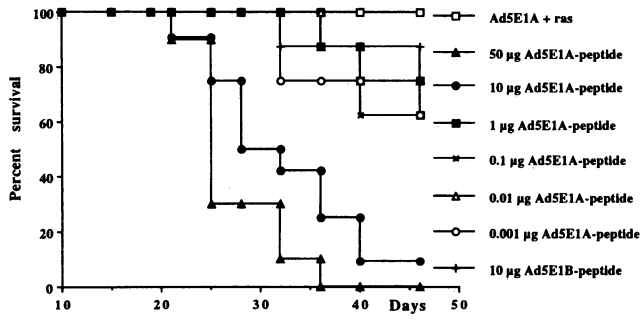

FIG. 2. Tumor growth of Ad5E1A + ras cells after titration of the Ad5E1A$_{234-243}$ peptide. Mice were immunized s.c. with irradiated Ad5E1A + ras cells, with 10 μg of the Ad5E1B$_{192-200}$-encoded control peptide (18) in IFA, or with 50 to 0.001 μg of the Ad5E1A peptide in IFA. Two weeks later mice were challenged with live Ad5E1A + ras cells on the other flank. Shown is the percentage of surviving animals.

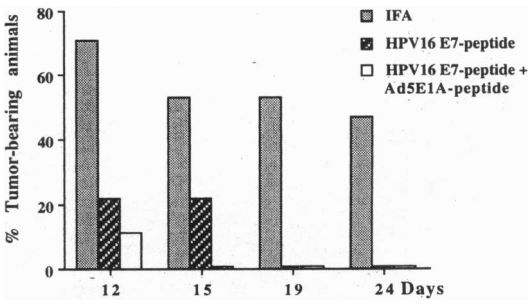

FIG. 3. Effect of immunization with the Ad5E1A$_{234-243}$ peptide is tumor-specific. Mice were immunized with 10 μg of the HPV16 E7$_{49-57}$ peptide in IFA, with a mixture of 10 μg HPV16 E7 peptide and 10 μg Ad5E1A peptide in IFA or with IFA only. Two weeks later mice were challenged with HPV16-transformed tumor cells. The HPV16 E7$_{49-57}$ peptide induces protective immunity against a challenge with HPV16 E7-transformed tumor cells (7). Mice immunized with the peptide mixture are also protected ($P = 0.03$, log-rank test). Shown is the percentage of tumor-bearing mice on several days after tumor challenge.

Table 1. Adoptively transferred Ad5E1A-specific CTL clone 5 is functionally deleted by the Ad5E1A peptide in vivo

	RMA + $E7_{49-57}$	RMA + $E1B_{192-200}$	RMA + $E1A_{234-243}$
Exp. 1			
Clone 5 + $E1B_{192-200}$	1 ± 1	2 ± 1	80 ± 10
Clone 5 + $E1A_{234-243}$	2 ± 1	4 ± 2	4 ± 2
Exp. 2			
Clone 5 + $E1B_{192-200}$	2 ± 1	8 ± 1	174 ± 21
Clone 5 + $E1A_{234-243}$	14 ± 2	17 ± 2	13 ± 2

Ad5E1A-specific CTL clone 5 (1.5×10^7) was adoptively transferred by intravenous injection to naive immuno-competent B6 mice at day 0. At day 3 these animals received s.c. the Ad5E1A peptide or the Ad5E1B peptide in IFA. At day 9 the spleen cells were taken and put in limiting dilution. At day 16 the contents of the wells were tested on syngeneic RMA cells loaded with 0.5 μM of the HPV16 $E7_{49-57}$-encoded control peptide, the $Ad5E1B_{192-200}$-encoded control peptide, or the $Ad5E1A_{234-243}$-encoded peptide. Values in table show minimum estimates of CTL ± SD per 10^6 spleen cells.

bined data on tumor outgrowth and Ad5E1A-specific CTL activity after peptide immunization indicate that both naive and preactivated Ad5E1A-specific CTL are functionally deleted by administration of the $Ad5E1A_{234-243}$-encoded CTL epitope.

The Ad5E1A Peptide Rapidly Diffuses Through the Body After s.c. Administration in IFA. The data described above indicate that intravenously injected Ad5E1A-specific CTL clone 5 is functionally deleted *in vivo* by s.c. administration of the Ad5E1A-encoded CTL epitope. CTL clone 5 is able to eradicate established Ad5E1-induced tumor in nude mice (14). To test if administration of the Ad5E1A-peptide leads to the inability of CTL clone 5 to eradicate established Ad5E1-induced tumors *in vivo*, we treated Ad5E1 tumor-bearing *nude* mice by intravenous injection of CTL clone 5. At the same time the Ad5E1A peptide, or as a control, the $Ad5E1B_{192-200}$-encoded peptide, was given s.c. together with rIL-2 in IFA. Unexpectedly, the animals that received CTL clone 5 and the Ad5E1A peptide died within 16 h (Table 2), whereas animals that received only the Ad5E1A peptide or CTL clone 5 together with the Ad5E1B peptide survived (Table 2). Mice that received an Ad5E1B-specific CTL clone in combination with the Ad5E1A peptide survived and eradicated the Ad5E1-induced tumors (data not shown). Similar findings were obtained in immunocompetent animals that received CTL clone 5 at the same time as the Ad5E1A peptide, together with rIL-2. Autopsy revealed that animals receiving the combination of

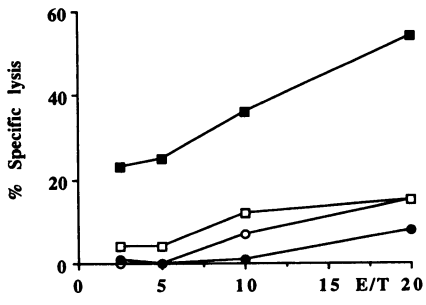

FIG. 4. Injection of the Ad5E1A peptide deletes naive Ad5E1A-specific CTL activity. Naive B6 mice, expressing a transgenic TCR specific for the Ad5E1A peptide, were injected with 10 μg of the $Ad5E1A_{234-243}$ peptide (open symbols) or 10 μg of the HPV16 $E7_{49-57}$ peptide (solid symbols) s.c. in IFA. Six days later 5×10^6 spleen cells of these animals were restimulated with 25% Ad5E1A peptide-loaded lipopolysaccharide blasts in a 24-well plate. After 5 days of culture the bulk CTL cultures were tested on untransformed B6 MEC (●, ○) or Ad5E1-transformed tumor cells (■, □). Shown is the percentage of specific lysis at different effector-to-target cell ratios.

Table 2. Combination therapy with adoptively transferred CTL clone 5 and Ad5E1A peptide leads to the death of mice.

Treatment	$Ad5E1A_{234-243}$, %	$Ad5E1B_{192-200}$, %
—	0	0
CTL clone 5, i.v.	100	0

Ad5E1-tumor-bearing *nude* mice (5 animals per group) were treated with 1.5×10^7 Ad5E1A-specific CTL clone 5 by intravenous (i.v.) injection. At the same day these mice received 10^5 Cetus units rIL-2 mixed with 100 μg peptide in IFA. Sixteen hours later all animals receiving Ad5E1A-specific CTL clone 5 and Ad5E1A peptide were dead. In all animals receiving CTL clone 5 and the Ad5E1B-encoded control peptide, tumors were eradicated. Values in table show percentage of dead mice.

CTL clone 5 and Ad5E1A peptide had severely congested lungs (data not shown). Apparently, the adoptively transferred Ad5E1A-specific CTL that are trapped in the capillary bed of the lungs become activated by injection of the Ad5E1A peptide, leading to the observed lung pathology. The mechanisms involved in induction of this lung pathology falls beyond the scope of this study and are now under further investigation. Nonetheless, these results show that s.c. administrated $Ad5E1A_{234-243}$ peptide in IFA rapidly diffuses throughout the body where it is recognized by Ad5E1A-specific CTL.

Protective Immunity Induced by Vaccination with Adenovirus. Immunization with the Ad5E1A peptide leads to Ad5E1A-specific CTL tolerance and severely affects the ability of B6 mice to cope with Ad5E1A-expressing tumors. Immunization with irradiated tumor cells, however, induces protective immunity against these tumor cells, showing the feasibility to induce protective immunity against Ad5E1A + ras transformed cells. Vaccination with irradiated tumor cells will, however, be difficult to execute on a large scale in a clinical setting. Recombinant adenoviruses might serve as efficient vaccine vehicles for the induction of protective antitumor immunity. Recombinant adenoviruses are currently being tested for their ability to deliver genes to a spectrum of nondividing cells *in vivo* for the treatment of genetic diseases. The use of recombinant adenoviruses is associated with transient gene expression due to a T-cell-mediated immune response against vector-derived proteins (23–25). This indicates that recombinant adenoviruses might also be used to induce protective immunity against tumors, when they contain DNA encoding for tumor antigens or tumor-derived T-cell epitopes. To test if adenoviruses can indeed be used to establish protective immunity against tumors, we immunized B6 mice with an adenovirus type 5 variant (*ts*149). Ad5*ts*149 is largely replication deficient, but expresses, upon infection, Ad5E1A-encoded protein (26). Mice immunized with Ad5*ts*149 showed high CTL reactivity against the Ad5E1A-encoded CTL epitope (Fig. 5*A* and *B*) and are protected against a subsequent challenge with Ad5E1A + ras cells (Fig. 5*C*). These results show the possibility to use recombinant adenoviruses expressing tumor antigens for the induction of tumor-specific protective immunity.

DISCUSSION

In the present report we show that a single s.c. immunizaton with 10 μg of the $Ad5E1A_{234-243}$-encoded CTL epitope rather than inducing protective immunity against a challenge of Ad5E1A-expressing tumor cells instead causes faster tumor outgrowth. This effect is peptide-specific and is rapidly induced. Ad5E1A-specific CTL activity can no longer be detected after injection of the Ad5E1A peptide from animals that express an Ad5E1A-specific transgenic TCR or that have received Ad5E1A-specific CTL clone 5 by adoptive transfer. Although the latter observation might be a consequence of an altered migratory pattern of the Ad5E1A-specific CTL clone,

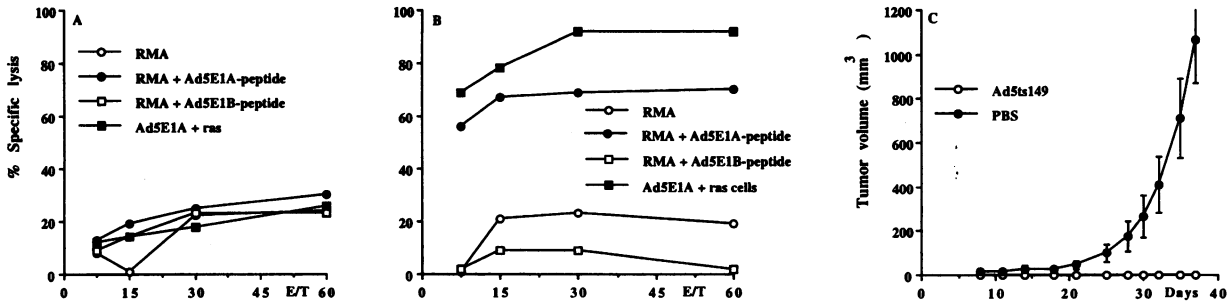

FIG. 5. Vaccination with a mutant adenovirus type 5, expressing Ad5E1A, induces Ad5E1A-specific CTL activity and protects against a subsequent challenge with Ad5E1A + ras cells. B6 mice were immunized (i.p.) with PBS + 0.5% BSA (*A* and *C*) or 2×10^9 plaque forming units of Ad5*ts*149 in PBS + 0.5% BSA (*B* and *C*). Two weeks after immunization, the spleen cells of these mice were restimulated *in vitro* with 10% Ad5E1A + ras cells (*A* and *B*), or the mice were challenged (s.c.) with Ad5E1A + ras cells (*C*). The lytic activity of bulk CTL were tested after 5 days of culture against Ad5E1A + ras cells, or syngeneic RMA cells loaded with 0.5 μM of the $Ad5E1B_{192-200}$-encoded control peptide or the $Ad5E1A_{234-243}$ peptide. Shown are the percentages of specific lysis at different effector-to-target cell ratios (*A* and *B*). The mean tumor volumes ± SEM ($n = 7$) of mice challenged with tumor cells are shown in mm^3 in *C*.

a more likely explanation is functional deletion of Ad5E1A-specific CTL. The Ad5E1A peptide rapidly diffuses throughout the body after s.c. injection in IFA and enhanced tumor outgrowth is also observed when the tumor challenge is given near the peptide–IFA depot in the same lymph nodes draining area (data not shown), indicating that the Ad5E1A-specific CTL are not massively attracted to the peptide–IFA depot.

Unlike mice immunized with adeno-derived peptides, mice immunized with live adenovirus or with irradiated tumor cells are protected against a subsequent challenge with Ad5E1A-expressing tumor cells. Great effort is currently directed toward the development of successful human gene therapies employing recombinant adenoviruses for gene transfer. One obstacle in obtaining stable expression of the delivered gene in the target cells using this method is a vector directed, T-cell-mediated immune response (23–25). By introducing tumor antigens or (several) tumor-encoded T-cell epitopes in these adenovectors it is possible to induce protective T-cell-mediated anti-tumor immunity as demonstrated here for the Ad5E1A epitope.

Peptide-based vaccination has been proven to induce T-cell-mediated protective immunity in several viral and tumor model systems (5–7, 9, 27). At the same time peptides have been found capable of T-cell tolerance induction (12, 13, 28–31). Repetitive and systemic (3 × i.p.) injections of high doses of peptide derived from LCMV-induced tolerance of LCMV-specific CTL. Local s.c. injections of this peptide protected mice against LCMV infection (13). Likewise, Kearney *et al.* (31) have shown in an adoptive transfer system that TCR-transgenic T cells are tolerized by systemic intravenous or i.p. injections of the specific peptide. In contrast, the same T cells are stimulated after s.c. injection of the peptide emulsified in complete Freund's adjuvants. They hypothesize that complete Freund's adjuvant not only functions as a depot that mediates a relatively slow release of peptide for a prolonged time period, but would also activate the local antigen presenting cells because it contains Mycobacterial components. We used as an adjuvant IFA. IFA functions as a noninflammatory vehicle because it lacks bacterial components. Nonetheless, s.c administration of the Ad5E1A peptide in complete Freund's adjuvant induces, in the Ad5E1A-tumor model, the same effects as injection of the peptide in IFA. The rapid, peptide-specific effect on the ability of B6 mice to control the outgrowth of Ad5E1A-expressing tumors is, like immunization in IFA, associated with severe lung congestion when given on the same day as an adoptively transferred Ad5E1A-specific CTL clone (data not shown). Induction of T-cell tolerance has also been described in other TCR transgenic mouse models. Continuous exposure to a high dose (100–150 μg) of peptide 366–374 deduced from the nucleoprotein of influenza virus of TCR transgenic mice expressing a TCR with a specificity for this peptide resulted in a depletion of most peripheral $CD8^+$ T cells bearing the transgenic TCR (28). This depletion appeared to be the result of thymic clonal elimination as well as peripheral loss of reactive T cells. Peripheral T cells in mice transgenic for TCR reactive with a simian virus 40-encoded peptide or a pigeon cytochrome *c* peptide are depleted *in vivo* by repeated injection of high doses (100 μg peptide injected i.p., three times) of the specific peptides (29, 30). In all these models, as opposed to the models where T-cell responses are induced, repetitive and systemic injections of relatively high doses of antigenic peptides are given. We now describe that a single s.c. injection of a much lower amount of peptide in IFA can induce T-cell unresponsiveness, leading to an enhanced outgrowth of tumors in normal immunocompetent animals.

The reasons why one peptide induces protective T-cell-mediated immunity and another T-cell tolerance when administered at comparable concentrations via the same vaccination scheme are intriguing. A possible explanation is that peptides administered s.c. in IFA eliciting protective immune responses, such as the HPV16 $E7_{49-57}$-encoded peptide are retained locally, forming a gradient of antigen. The Ad5E1A peptide diffuses rapidly throughout the body, instigating downregulation of the Ad5E1A-specific CTL response. Systemic distribution might lead to massive activation of peptide-specific CTL, that, especially if associated with inappropriate costimulation, may result in the clonal exhausting of these CTL. By analogy, it has been reported in an LCMV model that virus spread and CTL induction/exhaustion are closely linked (32). Infection with LCMV–DOCILE, a virus isolate that replicates rapidly and widely when injected in mice, induced LCMV-specific CTL tolerance, whereas infection with LCMV–WE, an isolate that replicates more slowly, induced long-lasting LCMV-specific CTL memory. In this respect it is remarkable to note that administration of the HPV16 $E7_{49-57}$ peptide on the same day as intravenous injection of a HPV16 $E7_{49-57}$-specific CTL clone does not lead to severe lung congestion (R.E.M.T., M.C.W. Feltkamp, M.P.M. Vierboom, C.J.M.M., and W.M.K., unpublished data), suggesting that the HPV16 E7-peptide is retained locally.

The observation that functional T-cell-specific tolerance can be induced by low doses of peptide administered locally is not only important for the development of safe peptide-based anti-tumor and anti-virus vaccines, but may also have implications for the design of therapeutic protocols for CTL-mediated autoimmune diseases and harmful T-cell responses against allogeneic organ transplants. Modification of $CD4^+$ T-cell-mediated autoimmune diseases and immunopathologies by peptides has been previously shown in a murine model of

experimental autoimmune encephalomyelitis and in a murine model of T-cell recognition of house dust mite allergens (33, 34). Similarly, it has been shown that oral administration of major histocompatibility complex-derived allopeptides can induce donor-specific T-cell tolerance (35, 36). By specifically tolerizing harmful CTL through injections with low doses of peptides it might be possible to prevent or temper CTL-dependent diseases.

In conclusion, in contrast to earlier reports describing induction of T-cell-mediated protection against a subsequent challenge of virus or tumor cells following a single s.c. injection of synthetic peptide in adjuvant, we now describe that such vaccination schemes can also lead to a functional deletion of tumor-specific CTL. Epitope delivery by vaccination with infectious adenovirus type 5 was associated with protection and CTL memory rather than tolerance and tumor outgrowth. Our results indicate that peptide-based vaccines must be employed with caution in a human setting, because immunization with tumor specific peptides might lead to a diminished rather than a protective immune response. However, in the Ad5E1A-tumor system only a single viral antigen is expressed. In naturally occurring tumors there may be a larger number of relevant T-cell epitopes and tolerance to a single epitope may not have the same dramatic consequences as observed in the Ad5E1A-tumor system. Nonetheless, candidate peptides for human anti-virus and anti-cancer vaccines should be tested for their immunizing or tolerizing properties *in vivo* in vaccination experiments in human leucocyte antigen-transgenic mice (10). In addition, investigation of immunizing and tolerogenic modes of peptide delivery deserves detailed attention.

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