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Multiple copies of a tumor epitope in a recombinant hepatitis B surface antigen (HBsAg) vaccine enhance CTL responses, but not tumor protection

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Abstract

We propose the replacement of endogenous epitopes with foreign epitopes to exploit the highly immunogenic hepatitis B surface antigen (HBsAg) as a vaccine vector to elicit disease-protective cytotoxic T-lymphocyte (CTL) responses. Locations were defined within the *HBsAg* gene where replacements of DNA encoding HBsAg epitopes may be made to generate functional recombinant (r) HBsAg DNA vaccines. We demonstrate that rHBsAg DNA vaccines encoding multiple copies of a model tumor epitope from human papillomavirus (HPV) elicit enhanced CTL responses compared to rHBsAg DNA vaccines encoding a single copy. We show that rHBsAg DNA vaccines elicit a marked prophylactic and long-lived therapeutic protection against epitope expressing tumor, although protective efficacy was not improved by increasing the number of copies of the tumor epitope DNA. These results demonstrate the efficacy of HBsAg as a vector for the delivery of foreign CTL epitopes using the epitope replacement strategy, and have implications for rHBsAg vaccine design. The results also have implications for the derivation of a therapeutic vaccine for HPV-associated squamous carcinoma.

Keywords: Recombinant DNA vaccine; Hepatitis B surface antigen; Cytotoxic T lymphocyte; Tumor protection; Human papillomavirus

Introduction

The hepatitis B virus (HBV) small envelope protein assembles with host-derived lipids into empty 22 nm virus-like particles (VLPs), consisting of 100–150 subunits of the ca. 226 amino acid hepatitis B surface antigen (HBsAg) molecule, without the participation of nucleocapsids. HBsAg VLPs induce potent antibody and cytotoxic T lymphocyte (CTL) responses when used for vaccination due to their particulate nature and repeated sub-unit structure (Schirmbeck et al., 1994; Schirmbeck and Reimann, 1996). HBsAg may also be delivered as a DNA vaccine, where CTL induction by intracellular translated HBsAg protein occurs through the 'classical' endogenous pathway as well as though the 'alternative' pathway via secreted HBsAg particles (Schirmbeck et al., 1995; Loirat et al., 2000). HBsAg vaccines administered as DNA or VLPs mimic the immunogenicity of live-attenuated vaccines, although they do not share most of their perceived disadvantages and ethical issues (e.g. threat to immunocompromised recipients, reversion to virulence).

While the intrinsic immunogenicity of HBsAg has been exploited as a carrier for the induction of antibody to foreign whole antigens or epitopes inserted at the C′ terminus (Berkower et al., 2004; Bisht et al., 2002; Baez-Astua et al., 2005), the N′ terminus (Vreden et al., 1991; Puaux et al., 2004; Bruss and Ganem, 1991) or in the hydrophilic 'a'-loop (Netter et al., 2001), the capacity of HBsAg to deliver foreign CTL epitopes is less well explored. Using a strategy of epitope replacement, we have recently demonstrated the powerful efficacy of HBsAg as a vector to deliver foreign CTL epitopes

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for the induction of insert-directed effector and memory CTL responses (Woo et al., 2006). We reasoned that this approach would predispose to the maintenance of spatial requirements for correct conformation of the HBsAg molecule, and also to the maintenance of appropriate antigen processing. Thus by deletion of an individual endogenous HBsAg CTL epitope and insertion of an individual foreign CTL epitope at the site of deletion, we recently demonstrated the induction of protective CTL responses for an infectious disease and for a carcinoma (Woo et al., 2006).

A crucial question for the delivery of foreign CTL epitopes by HBsAg vector is the amount of foreign epitope which must be delivered to ensure a maximal response. In the present study, we define five locations at which deletions of DNA encoding endogenous CTL epitopes may be made and replaced with DNA encoding a foreign tumor-protective CTL epitope to generate a panel of recombinant (r) HBsAg DNA vaccines. We investigate the efficacy of induction of CTL responses and disease protection by rHBsAg DNA vaccines encoding a single copy versus multiple copies of the tumor-protective CTL epitope.

We used an epitope of human papillomavirus (HPV) 16 E7 protein as a model tumor antigen. HPVs are associated with a number of squamous cell cancers, most notably cervical carcinoma. The oncogenic HPV E7 protein is responsible for transforming epithelial cells by mechanisms that require E7 to persist for cells to remain transformed (Tommasino and Crawford, 1995; von Knebel et al., 1994). Thus, E7 is a tumor associated antigen (TAA) to which CTL-inducing immunoprotective strategies may be directed. $CD8^+$ cytotoxic T lymphocytes (CTLs) are crucial components of protective immune responses to E7-associated tumors (von Knebel et al., 1994).

We demonstrate that the *HBsAg* gene contains (at least) five sites at which DNA encoding endogenous HBsAg CTL epitopes may be excised, and replaced with DNA encoding a E7 CTL epitope to elicit powerful effector and memory CTL responses when administered as recombinant DNA vaccines. We show that the rHBsAg DNA vaccine elicits potent prophylactic and long-lived therapeutic protection against E7-expressing tumor. We show that when used at a standard doses, rHBsAg DNA encoding multiple copies of the E7 epitope elicit superior CTL responses measured *in vitro* compared to rHBsAg DNA vaccine coding a single copy, though these enhanced responses were not associated with improved tumor protection.

Our findings underscore the efficacy of HBsAg as a vector for the delivery of foreign CTL epitopes, using the epitope replacement strategy. They address the potency of rHBsAg vaccines containing single versus multiple copies of a foreign epitope, and so have generic implications for rHBsAg vaccine design. The results also have specific implications for the derivation of a therapeutic vaccine for HPV-associated squamous carcinomas.

Results

Immunization with rHBsAg DNA encoding a foreign CTL epitope inserted at any one of five locations elicits insert-directed CTLs

A number of HBsAg CTL epitopes in transmembrane and internal regions of the HBsAg molecule restricted through murine (Schirmbeck et al., 1995, 2002) and human (Loirat et al., 2000) MHC class 1 molecules have been described. In a first set of experiments, we created 5 constructs in each of which one of the more immunogenic of these HBsAg-specific CTL epitopes (VLQ, IPQ, FLG, GLS and SIL; Fig. 1 and Table 1) was deleted and replaced with the tumor-protective CTL epitope RAH (^{49}RAHYNIVTF^{57}) from the E7 protein of HPV16 (Feltkamp et al., 1993). Thus, five recombinant HBsAg DNA vaccines were derived, each containing a single copy of the RAH minigene. We asked whether these rHBsAg DNA vaccines would elicit RAH-directed effector and memory CTL responses.

To investigate effector responses we quantified epitope-specific interferon-gamma (IFN-γ) secretion of *ex vivo* splenocytes by ELISPOT assay. *Ex vivo* splenocytes from groups of mice, each group immunized with one of the five constructs encoding a RAH minigene inserted at different locations (Fig. 2a), secreted IFN-γ when cultured *in vitro* with, but not without, RAH peptide (Fig. 2b). *Ex vivo* splenocytes from mice immunized with HBsAg wild type (W/T) DNA cultured with RAH peptide did not secrete IFN-γ above the level observed when cultured without peptide (data not shown).

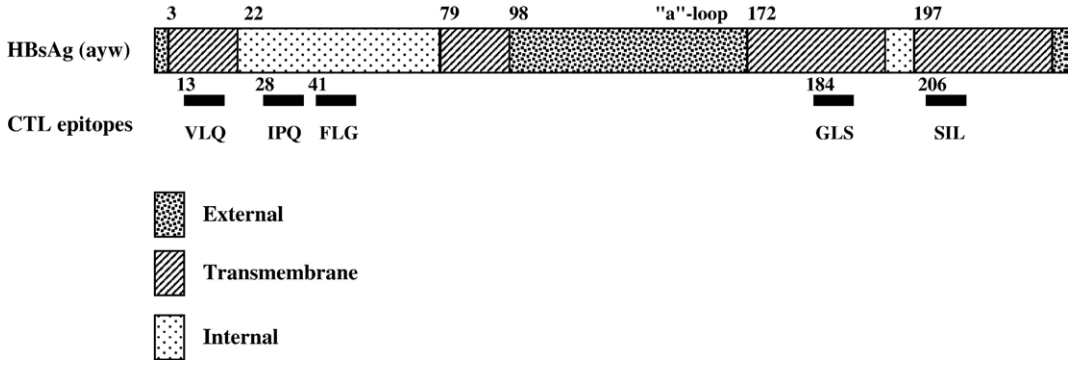

Fig. 1. Schematic of the 226 amino acid HBsAg-s molecule depicting the location of five endogenous CTL epitopes, (black boxes) located in the transmembrane and internal domains (see Table 1 for details of epitopes).

Table 1
HBsAg CTL epitopes

Designation	Sequence	MHC restriction	Reference
VLQ	^{13}VLQAGFFLL^{21}	HLA A*0201	Chisari and Ferrari (1995)
IPQ	^{28}IPQSLDSWWTSL^{39}	H-2L^{d}	Ando et al. (1993)
FLG	^{41}FLGGTPVCL^{49}	HLA A*0201	Chisari and Ferrari (1995)
GLS	^{184}GLSPTVWLSV^{193}	HLA A*0201	Nayersina et al. (1993)
SIL	^{206}SILSPFLPLL^{215}	HLA A*0201	Chisari and Ferrari (1995)

To investigate memory responses, we asked whether immunization with each of these five constructs expressing a RAH minigene inserted at different locations would induce CTL following restimulation, which would kill target cells expressing the RAH CTL epitope. Splenocytes from DNA-immunized mice were restimulated *in vitro* for 6 days with RAH peptide, and reacted with peptide-pulsed H-2^{b} target cells. Restimulated splenocytes from all the immunized groups of mice killed RAH peptide-pulsed targets (Fig. 2c). Notably the efficiency of killing was comparable with that seen in identically restimulated splenocytes from mice immunized with high dose RAH peptide plus adjuvant (Fig. 2c). Immunization with high-dose CTL epitope peptide plus adjuvant is a powerful inducer of CTL responses and a 'standard' against which other CTL-inducing modalities may be compared (Doan et al., 1998).

Taken together, these data indicate that replacement of DNA encoding HBsAg-specific CTL epitopes with DNA encoding a foreign CTL epitope at specific locations within the HBsAg molecule to generate recombinant DNA immunogens, engenders effector and memory CTL responses to the foreign epitope.

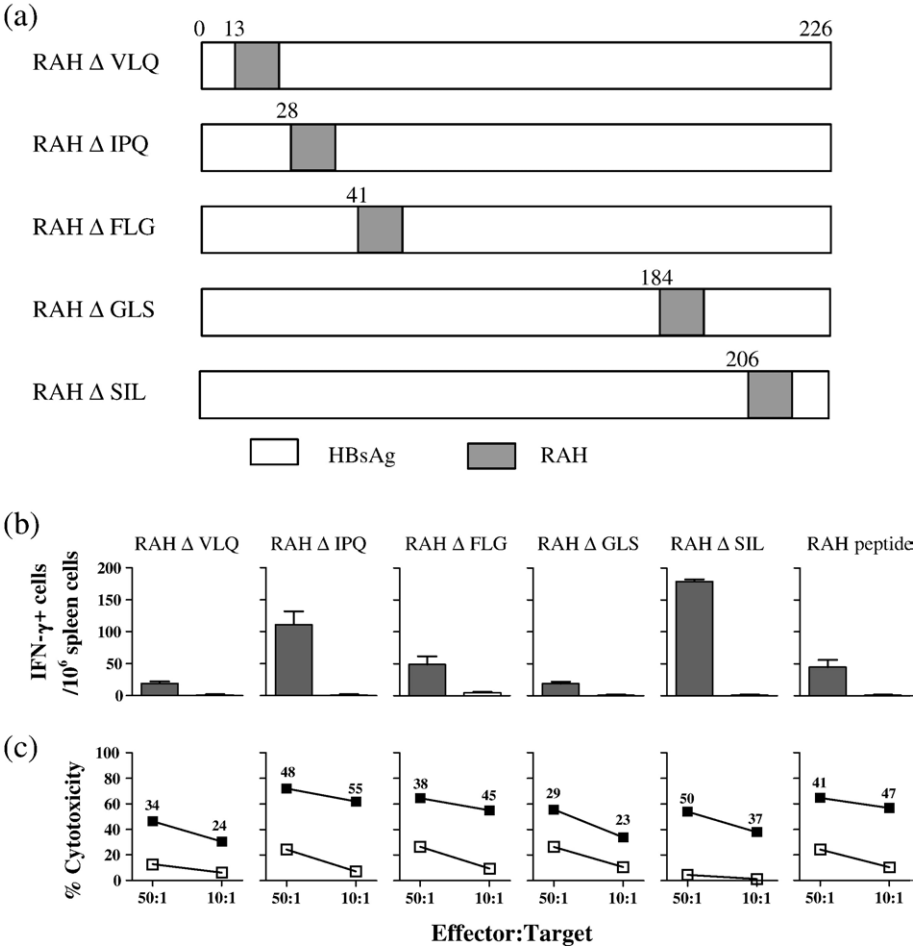

Fig. 2. (a) Schematic of chimeric HBsAg DNA constructs in each of which an endogenous CTL epitope (Fig. 1) was deleted and replaced with a single copy of the HPV E7 CTL epitope RAH. (b, c) Immunization with chimeric HBsAg DNAs encoding a single copy of RAH elicits RAH-directed CTL responses. Groups of mice (3 per group) were immunized twice i.d. with DNA or once s.c. with RAH peptide plus adjuvant. (b) Effector IFN-γ-secreting cells were quantified by ELISPOT assay using splenocytes harvested at 14 days (DNA immunizations) or 10 days (peptide immunization) after immunization, and incubated for 15–18 h. with RAH peptide (left histogram bar) or without peptide (right histogram bar), as shown. Bars represent means ± standard deviation of 3 replicates. (c) Epitope-specific CTL memory responses. Groups of mice were immunized as above. Percent cytotoxicity of splenocytes restimulated for 6 days *in vitro* with RAH peptide was measured in a ^{51}Cr release assay using EL4.A2 target cells pulsed with RAH peptide or without peptide as shown. Data points represent means of 3 replicates ± standard deviation (standard deviations were always less than 3%).

Immunization with rHBsAg DNA encoding multiple copies of an inserted foreign epitope elicits improved insert-directed CTL responses

We asked whether rHBsAg DNA vaccines encoding multiple copies of a foreign CTL epitope would elicit superior CTL responses compared to a rHBsAg DNA vaccine encoding a single copy of the foreign epitope. Recombinant HBsAg DNAs were constructed to encode three, four and five copies of RAH CTL epitope (RAHx3, RAHx4, and RAHx5; Fig. 3a), at locations individually validated to express inserted RAH for immune response induction (Fig. 2a). IFN-γ ELISPOT assays were conducted on splenocytes harvested *ex vivo*, and also after a further 6-day restimulation *in vitro* with RAH peptide, from groups of mice immunized with the four constructs expressing one, three, four or five RAH minigenes. Both *ex vivo* (Fig. 3b) and post-restimulation (Fig. 3c) splenocytes from mice immunized with rHBsAg DNA encoding one or more copies of the RAH minigene secreted substantial amounts of IFN-γ when cultured with, but not without, RAH peptide. The numbers of secreting splenocytes from mice immunized with RAHx3, RAHx4 and RAHx5 were significantly higher than splenocytes from mice immunized with RAHx1.ΔGLS (*ex vivo* splenocytes, $p<000.1$; post-restimulation splenocytes $p<0.001$). The numbers of IFN-γ secreting splenocytes from mice immunized with RAHx3, RAHx4 and RAHx5 did not differ significantly from each other (*ex vivo*, $p>0.5$; post-restimulation $p>0.7$). *Ex vivo* splenocytes from mice immunized with pHBsAg not encoding RAH (RAHx0) did not secrete IFN-γ above background when cultured with RAH peptide (Figs. 3b, c).

Together these data indicate that immunization with rHBsAg DNA vaccines encoding multiple (three, four or five) copies of a foreign CTL epitope elicits superior effector (*ex vivo*) and memory (post-restimulation) CTL responses, compared with a rHBsAg DNA vaccine encoding a single copy of the foreign CTL epitope. The data also indicate that provision of four or more copies of the foreign minigene in rHBsAg DNA does not augment the responses seen by provision of three copies.

We also measured RAH-directed memory CTL responses by ^{51}Cr release cytotoxicity assay. Restimulated splenocytes from mice immunized with rHBsAg vaccines encoding single or multiple copies of RAH epitope specifically killed RAH expressing target cells (Fig. 3d).

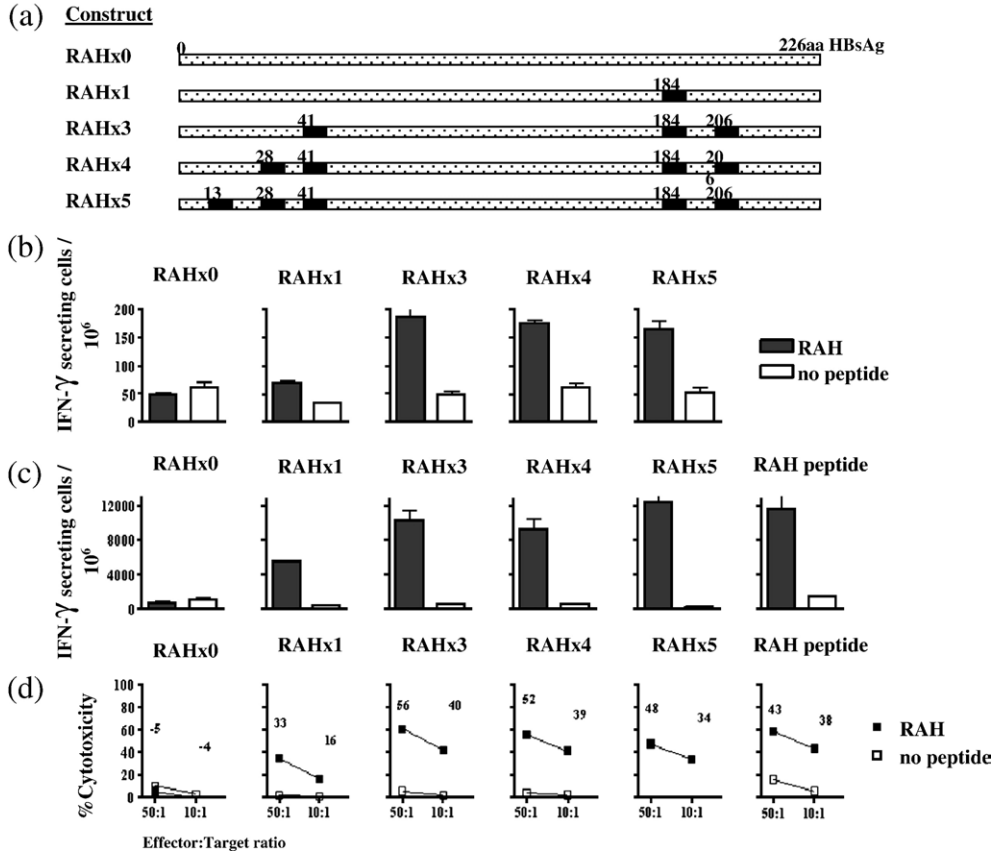

Fig. 3. (a) Schematic of rHBsAg DNA constructs in which 1, 3, 4 or 5 endogenous CTL epitopes (see Table 1 and Fig. 1) were deleted and replaced with the HPV E7 CTL epitope RAH. (b–d) Immunization with chimeric HBsAg DNAs encoding RAH elicits RAH-directed CTL responses. Mice (3 per group) were immunized twice i.d. with DNA or once s.c. with RAH peptide plus adjuvant (b, c). IFN-γ-secreting splenocytes harvested at 14 days (DNA immunizations) or 10 days (peptide immunizations) after immunization, were quantified by ELISPOT assay either *ex vivo* (b) or after 6 days of restimulation *in vitro* with RAH peptide (c). Splenocytes were incubated for 15–18 h. with RAH peptide or without peptide, as shown. Bars represent means±standard deviation of 3 replicates. (d) Groups of mice were immunized as above. Percent cytotoxicity of splenocytes restimulated with RAH peptide was measured in a ^{51}Cr release assay using EL4.A2 target cells pulsed with RAH peptide or without peptide as shown. Data points represent means of 3 replicates±standard deviation. (Standard deviations were always less than 3%.)

Immunization with rHBsAg DNA encoding multiple copies versus a single copy of a tumor epitope does not significantly improve tumor prevention

The HPV 16 E7 oncoprotein is necessary for the maintenance of the transformed phenotype in HPV-associated squamous cancers (von Knebel et al., 1994), and so E7 functions as a tumor specific antigen to which immunomanipulative therapies may be directed (Tindle, 2002; Frazer, 2004). RAH-directed CTL protect against the growth of HPV16 E7 expressing tumors in H-2^b mice (Feltkamp et al., 1993) thus providing a model of HPV-associated tumor protection in humans. We asked whether immunization with rHBsAg DNA vaccines encoding three copies of the RAH epitope would provide superior protection against subsequent challenge with E7-expressing TC-1 tumor (Lin et al., 1996) compared to immunization with rHBsAg DNA vaccines encoding a single copy. As a single copy vaccine, we used the rHBsAg construct in which the RAH tumor epitope was inserted at the C′ terminal ΔGLS site (RAHx1.ΔGLS, Fig. 2(a)). Two of seven mice per group immunized with RAHx0, RAHx1.ΔGLS, or RAHx3, were sampled to confirm induction of RAH-directed effector CTL by IFN-γ ELISPOT assay (Fig. 4a). In agreement with the above findings, mice immunized with rHBsAg DNA encoding three copies of RAH elicited a significantly greater effector response than mice immunized with rHBsAg encoding a single copy of RAH (Fig. 4a; $p<0.001$). The remaining five mice per group were challenged with TC-1 tumor cells, and tumor growth monitored over a 24-day period. Mice were euthanized when tumors reached 500 m^3 in area (in accord with ethical guidelines). 80% and 100% of mice immunized with RAHx1.ΔGLS and RAHx3 respectively survived to 24 days (difference not significant; $p>0.05$). In contrast, significantly fewer (20%) of the mice immunized with RAHx0 survived to day 24 ($p<0.04$) (Fig. 4b). These data indicate that immunization with rHBsAg DNA vaccines encoding the RAH epitope elicits CTL responses associated with protection against establishment of E7-expressing tumor, and that rHBsAg DNA encoding one copy of RAH is not significantly less effective in invoking protection than rHBsAg DNA encoding three copies, at the doses used.

Immunization with rHBsAg DNA encoding multiple copies versus a single copy of a tumor epitope does not significantly improve tumor therapy

We evaluated the efficacy of rHBsAg DNA encoding one copy (RAHx1.ΔIPQ and RAHx1.ΔSIL, Fig. 3a) and three copies (RAHx3) of the RAH epitope as therapeutic vaccines for

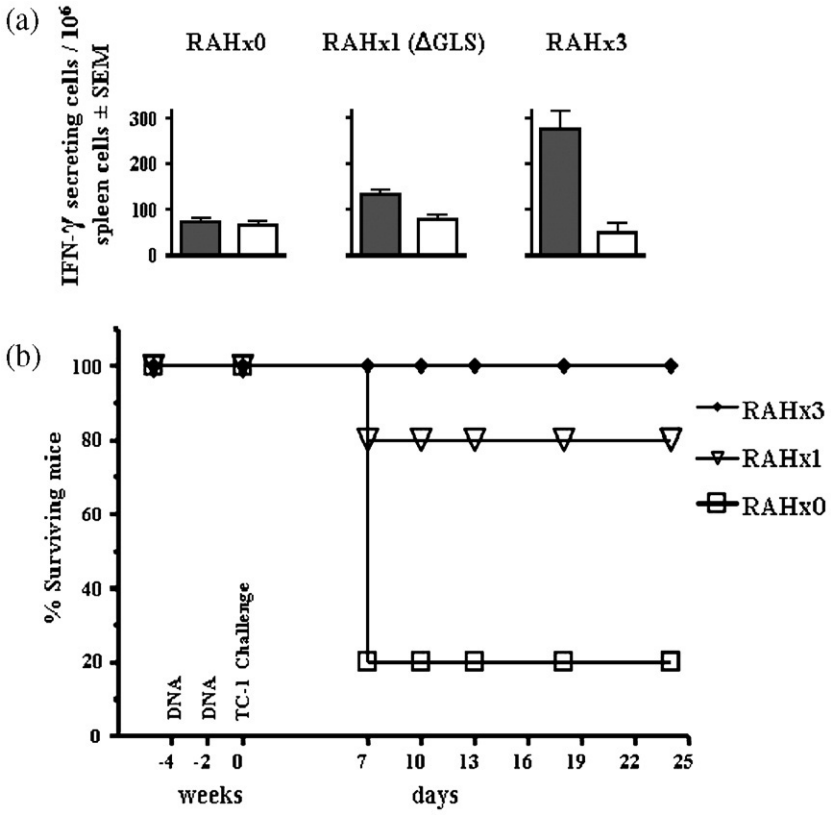

Fig. 4. Recombinant HBsAg DNA encoding one or three copies of the RAH CTL epitope of HPV16 E7 protects against challenge with E7-expressing tumor. (a) Mice (7 per group) were immunized twice i.d. with RAHx0, RAHx1.ΔGLS, RAHx3. IFN-γ-secreting splenocytes harvested at 14 days from two representative mice per group were quantified by ELISPOT assay with or without RAH peptide as shown. Bars are means ± standard deviation of 3 replicates. (b) Growth of E7-expressing tumor in immunized mice and controls. The remaining seven mice per group were challenged with 2×10^5 E7-expressing TC-1 tumor cells 14 days after the second immunization, and tumor growth monitored as percentage of mice with palpable tumor. Mice with tumors exceeding 500 mm^3 were euthanized in accordance with animal ethics requirements. Results are expressed as surviving mice (%) at the indicated time points.

the resolution of E7 tumor. Groups of mice were inoculated with TC-1 tumor cells. Commencing 3 days later, mice were immunized three times with rHBsAg DNA vaccines encoding one or three copies of the RAH epitope (Fig. 5). 14 days after the final immunization, three representative mice of thirteen per group immunized with RAHx1.ΔIPQ, RAHx1.ΔSIL, or RAHx3 were sampled to confirm induction of RAH-directed effector CTL by IFN-γ ELISPOT assay. As expected, mice immunized with rHBsAg DNA encoding three copies of RAH displayed a significantly greater effector response than mice immunized with rHBsAg encoding a single copy of RAH ($p<0.001$) (Fig. 5a). In addition, we confirmed that restimulated splenocytes from the representative mice immunized with rHBsAg DNA encoding the RAH epitope specifically killed target cells pulsed with RAH peptide, and target cells expressing whole E7 protein (Fig. 5b).

Palpable tumor incidence was followed to 80 days. By day 11, tumors were palpable in 60% of mice immunized with wild-type HBsAg DNA (RAHx0). In contrast, palpable tumor onset was delayed in mice immunized with rHBsAg DNA vaccines encoding the RAH epitope. Significantly fewer (30% or less; $p<0.05$) of the mice immunized with rHBsAg DNA vaccines encoding one or three copies of the RAH epitope developed palpable tumor. There was no significant difference in tumor incidence among groups of mice immunized with the vaccines encoding one copy of RAH, or three copies of RAH ($p>0.8$) (Fig. 5c).

Tumor volumes were recorded. Beyond 20 days tumors were significantly smaller in all groups of mice immunized with rHBsAg DNA vaccines encoding the RAH epitope compared with mice immunized with wild-type HBsAg DNA ($p<0.001$) (Fig. 5d). There was no significant difference in tumor volumes between mice immunized with rHBsAg encoding a single copy of RAH epitope (RAHx1.ΔIPQ and RAHx1.ΔSIL) and mice immunized with rHBsAg encoding three copies of RAH epitope ($p>0.7$).

We also examined whether immunization would retard the growth of well-established palpable tumors. Groups of nine mice with TC-1 tumors measuring at least 80 mm^3 were immunized with rHBsAg DNA vaccines encoding one copy (RAHx1.ΔSIL) or three copies of the RAH epitope. Both these RAH-encoding vaccines slowed the growth of tumor compared to wild type (W/T) HBsAg vaccine when measured at 19 days, although the difference was barely significant ($P=0.049$). There was no significant difference in degree to which tumor growth was slowed by rHBsAg vaccine encoding one copy of RAH or the rHBsAg vaccine encoding three copies of RAH ($p>0.3$) (Fig. 6).

Mice cured of tumor by therapeutic immunization with tumor epitope-encoding rHBsAg vaccines retain long-term anti-tumor immunity

We examined whether mice which were cured of their tumors by immunization with rHBsAg DNA encoding one or multiple copies of RAH tumor epitope retained long term RAH-directed CTL responses and resistance to the growth of a subsequent challenge with TC-1 tumor. Representative mice which had been inoculated with TC-1 tumor cells and subsequently therapeutically immunized with chimeric HBsAg DNA vaccines encoding one or three copies of RAH epitope to resolve tumor (see Fig. 5, above), were evaluated at 100 days for RAH-directed memory CTL responses. IFN-γ ELISPOT assays were conducted on splenocytes after restimulation *in vitro*. Post-restimulation splenocytes from mice immunized with rHBsAg DNA encoding one or three copies of RAH secreted substantial amounts of IFN-γ when cultured with, but not without, RAH peptide (Fig. 7a). The numbers of IFNγ-secreting splenocytes from mice immunized with RAHx3, was significantly higher than the number IFNγ-secreting splenocytes from mice immunized with rHBsAg encoding a single copy of RAH (RAHx1.ΔIPQ or RAHx1.ΔSIL) ($p<0.001$) in accord with findings reported in Fig. 3c. We also measured RAH-directed CTL responses by ^{51}Cr cytotoxicity assays. Restimulated splenocytes from mice immunized with HBsAg encoding a single copy of RAH or with RAHx3 showed high levels of cytotoxicity towards RAH-expressing target cells (Fig. 7b).

The remaining members of the groups of mice previously cured of tumor by rHBsAg DNA immunization >100 days earlier were rechallenged with 2×10^5 TC-1 tumor cells. One hundred percent of mice which had previously received RAHx1.ΔIPQ, RAHx1.ΔSIL or RAHx3 vaccines >100 days earlier were protected against the growth of tumor, whereas all of a group of unimmunized mice developed tumors.

Together these results demonstrate that mice cured of previous tumor by therapeutic immunization with rHBsAg DNA vaccines encoding either a single copy or three copies of RAH epitope retain RAH-directed memory CTL responses, and are protected against subsequent tumor challenge, for at least 100 days.

Discussion

The adaptive cellular arm of the immune response plays a major role in the control of tumors and pathogens and the response is mediated largely by $CD8^+$ CTLs. There are difficulties in deriving vaccines capable of delivering multiple CTL epitopes which are acceptable for humans (Gupta and Siber, 1995). CTL epitopes delivered as peptide mixes have generally proved ineffective, may require a source of CD4 'help', and are hampered at the regulatory level by restriction on the use of adjuvants capable of inducing effective cellular responses. Vaccines containing recombinant tumor- and pathogen-derived proteins are difficult to construct, and are further limited by availability of acceptable vector systems for use in humans.

HBsAg, already licensed as the human vaccine for hepatitis B virus (HBV) infection, is an attractive vector for the delivery of epitopes from tumors and other pathogens, from both a regulatory and scientific point of view (Davis et al., 1993; Schirmbeck et al., 1994). While HBsAg has been used experimentally as a carrier for foreign B cell epitopes for antibody induction, there have been few reports exploiting the inherent immunogenicity of HBsAg to deliver foreign CTL

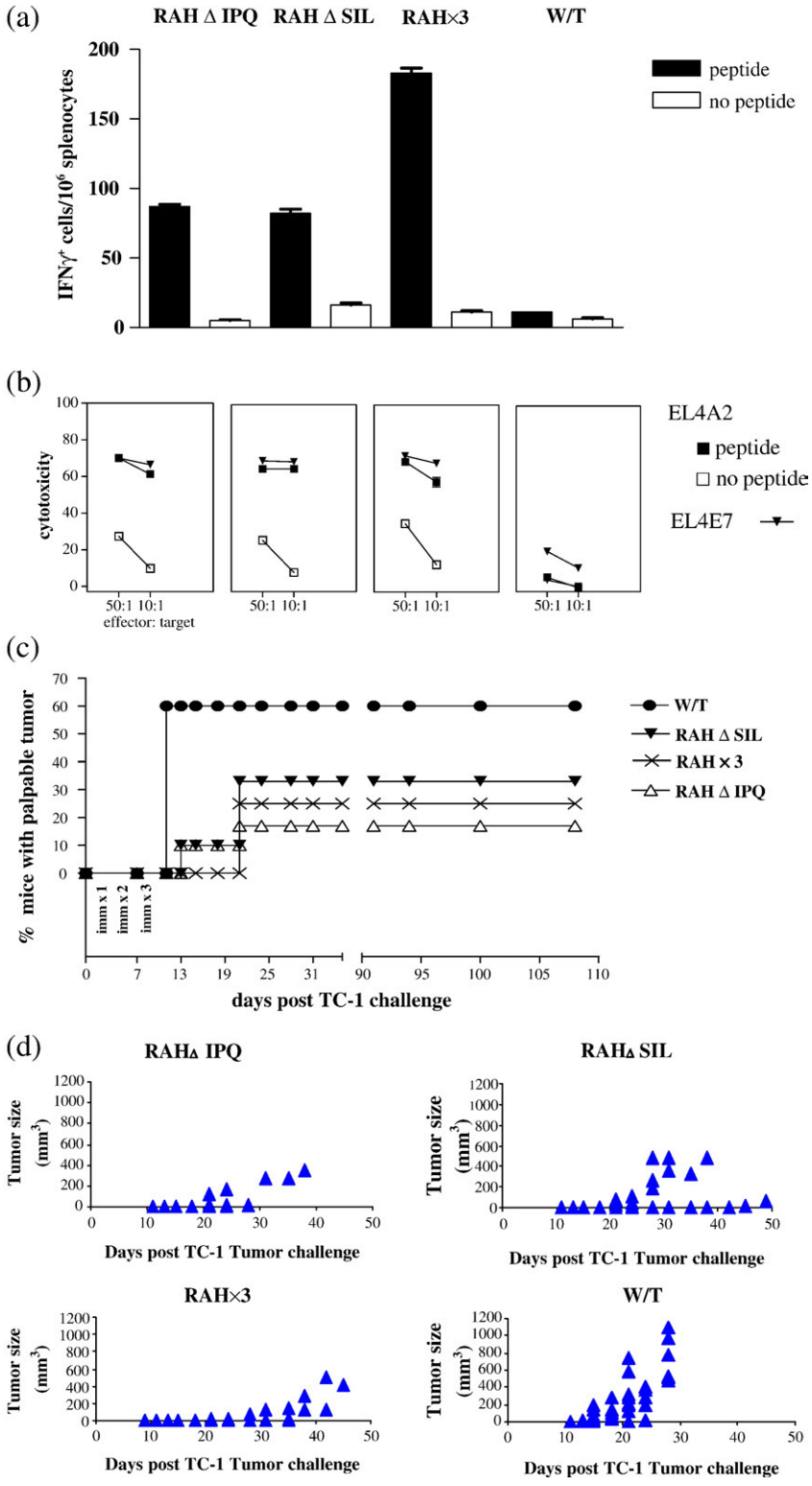

Fig. 5. Recombinant HBsAg DNA vaccines encoding one or three copies of the RAH CTL epitope of HPV16 E7 protect against the growth of pre-existing E7-expressing tumor. Five groups of mice (13 per group) were injected s.c. with 5×10^4 TC-1 tumor cells and the tumor allowed to growth for 3 days. The groups were then immunized 3 times at 4-day intervals with either RAHx0, RAHx1ΔIPQ or RAHx1ΔSIL; (Fig. 2a) or with RAHx3, and subsequent tumor growth quantified. To confirm that immunized mice were mounting E7-directed CTL responses, IFN-γ-secreting splenocytes harvested from three representative mice per group 6 day after the final immunization were quantified by ELISPOT assay with or without RAH peptide (a), or restimulated *in vitro* and reacted with RAH-expressing target cells in ^{51}Cr release assay (b). Bars are means ± standard deviation of 3 replicates. Tumor growth was followed in the remaining mice and expressed as % mice with palpable tumor at the indicated time points (c), and as tumor volumes (mean ± S.E.M.) of those mice with persisting tumor (d).

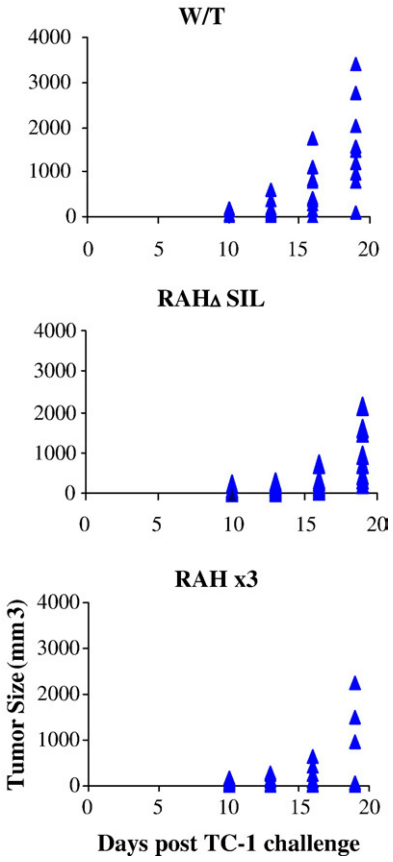

Fig. 6. Recombinant HBsAg DNA vaccines encoding one or three copies of the RAH CTL epitope of HPV16 E7 protect against the growth of well-established E7-expressing tumor. Groups of mice with TC-1 tumors measuring 80 mm^3 were immunized with rHBsAg DNA encoding 1 copy (RAHx1.ΔSIL) or three copies (RAHx3) of RAH epitope. Tumor volumes of individual mice were recorded at intervals to 19 days.

epitopes (e.g. Fomsgaard et al., 1999; Bryder et al., 1999; Michel et al., 2007). We have demonstrated that tumor- and pathogen-associated CTL epitopes may be genetically incorporated into HBsAg by replacement of two endogenous CTL epitopes (IPQ and GLS) which when delivered as plasmid DNA vaccines elicit CTL-associated disease protective responses (Woo et al., 2006).

The vaccine for HBV is administered as antibody-inducing HBsAg virus-like particles. However, data suggests that DNA may be the preferred modality for CTL induction (Davis et al., 1996, 1997). That a single low-dose injection of HBsAg plasmid DNA gives CTL detectable for many months post vaccination (Davis et al., 1993; Edgtton, unpublished data) suggests that continuous exposure of small doses of antigen provided from on-going DNA transcription may be necessary to maintain immunity. Such priming with prolonged exposure to antigen predisposes the response in the direction of 'central memory' T cells (T_{CM}) (Wherry et al., 2005) and enables conversion of effector memory T cells (T_{EM}) to T_{CM} (Wherry et al., 2003), which afford greater protection against tumors (Klebanoff et al., 2005) and viral infection (Wherry et al., 2003; Zanetti and Franchini, 2006). A variety of cells are likely to be transfected by injected HBsAg DNA, though only transfection of "professional" antigen presenting cells (APCs) leads to activation of the CTL response via the intracellular (endogenous) processing pathway (Sbai et al., 2002). VLP formation within the APC is not an essential requirement for this to occur (Sbai et al., 2002). Secretion of HBsAg VLPs (e.g. by DNA-transfected muscle cells) allows uptake of secreted particles by APCs for processing via the 'exogenous pathway' (Davis et al., 1993), thus providing a second mechanism for HBsAg CTL generation (Schirmbeck and Reimann, 2002). Thus the strong CTL responses induced by HBsAg DNA vaccination, requiring ca. 2500-fold lower dose than conventional DNA vaccines (Roy et al., 2000), may be explained in terms of prolonged and higher expression, secretion for uptake of VLPs by APC, superior antigen processing and the presence of multiple T-helper epitopes in the HBsAg backbone.

In the current study, we used the epitope replacement strategy developed in our laboratory (Woo et al., 2006) to extend to five sites (ΔVLQ, ΔIPQ, ΔFLG, ΔGLS and ΔSIL: Fig. 1) where DNA encoding endogenous HBsAg CTL epitopes could be deleted and replaced with DNA encoding a foreign CTL epitope (from the E7 oncoprotein of HPV16) to derive five rHBsAg DNA vaccines (Fig. 2a). Each of these vaccines elicited E7-directed effector and memory CTL responses, though with

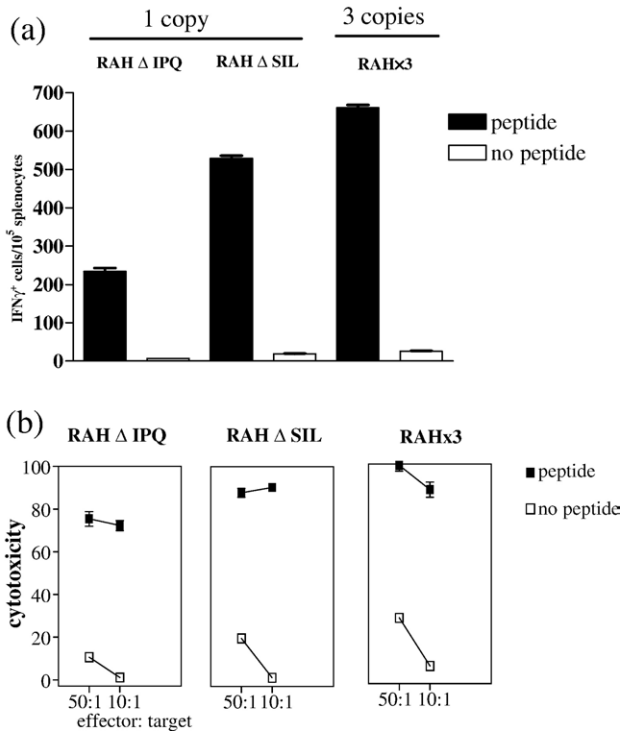

Fig. 7. Longevity of anti-tumor response. Groups of mice were cured of TC-1 tumor by three rHBsAg immunizations with rHBsAg containing 1 copy of RAH (RAHx1.ΔIPQ or RAHx1.ΔSIL) or 3 copies of RAH (RAHx3). RAH-directed memory CTL responses were evaluated in two representative mice per group. Splenocytes harvested from two representative mice per group were restimulated for 6 days *in vitro* with RAH peptide and quantified by ELISPOT assay with or without specific peptide (RAH) (a), or by ^{51}Cr release assay (b). Bars are means ± standard deviation of 3 replicates.

varying efficiency (Figs. 2b, c). Our data do not address the cause of this variation though it likely relates to translational efficiency, and/or efficacy of antigen processing and presentation, of the various HBsAg recombinants.

Since the magnitude of a primary T cell response parallels antigen dose (Wherry et al., 1999; Kaech and Ahmed, 2001) and the pool of memory T cells surviving apoptosis reflects the magnitude of the primary expansion (Wherry et al., 1999), we were interested to determine whether provision of multiple copies of the inserted minigene encoding the RAH tumor epitope in rHBsAg DNA vaccines would augment the tumor epitope-directed CTL response. Thus, we constructed a panel of rHBsAg DNA vaccines encoding multiple copies of the tumor epitope, each copy at one of the above predetermined sites (Figs. 2 and 3a), and investigated the immunogenicity of these vaccines. Since our earlier data demonstrated that two copies of a minigene encoding a foreign epitope (from RSV) did not significantly improve immunogenicity over a single copy (Woo et al., 2006), we elected in the present study to examine vaccines encoding three copies or more of a foreign epitope (from HPV). To address whether insert at each of the sites was immunogenic in the context of multicopy rHBsAg vaccines we inserted DNA encoding five *different* foreign CTL epitopes into a single rHBsAg vaccine, one epitope at each of the five sites to show that this rHBsAg DNA vaccine elicited CTL to each of the encoded foreign CTL epitopes simultaneously (data not shown).

We demonstrate that immunization with rHBsAg DNA vaccines encoding multiple (three, four or five) copies of the RAH tumor epitope elicits superior effector and memory CTL responses compared with a rHBsAg DNA vaccine encoding a single copy of the foreign CTL epitope. This conclusion held when the tumor epitope was encoded at any of three sites (ΔGLS, ΔIPQ, or ΔSIL) in a single copy construct (Figs. 3, 5, and 7). The data also indicate that four or five copies of the tumor epitope DNA encoded in rHBsAg did not further improve the CTL responses elicited by rHBsAg encoding three copies (Fig. 3).

We examined the capacity of rHBsAg DNA vaccines encoding a single copy or three copies of the tumor epitope to afford tumor protection. We found that the weaker effector CTL response induced by rHBsAg encoding a single copy (Fig. 4a) was nonetheless associated with a potent protective response to challenge with E7-expressing tumor (Fig. 4), similar to the protective response induced by immunization with rHBsAg encoding three copies, where the effector CTL response was significantly stronger. These novel data also demonstrate that rHBsAg DNA vaccine encoding a single copy of the RAH tumor antigen inserted at the C′ terminus (RAHx1.ΔGLS) is as effective at mediating tumor prevention as a rHBsAg DNA vaccine encoding a single copy of the RAH tumor antigen inserted at the N′ terminus (RAHx1.ΔIPQ) (Woo et al., 2006). Furthermore we extend these and our previous findings on tumor prevention (Woo et al., 2006) to demonstrate that the weaker effector CTL response induced by immunization of tumor-bearing mice with rHBsAg encoding a single copy of the tumor epitope did not lessen the therapeutic effect afforded by immunization with rHBsAg encoding three copies of the tumor epitope (Figs. 5a, c). Immunization with rHBsAg encoding a single copy or three copies of the tumor antigen were both associated with powerful memory cytotoxic responses in these mice which specifically killed both epitope-pulsed target cells and target cells constitutively expressing whole tumor antigen (E7) (Fig. 5b). Furthermore, mice cured of their tumors 100 days previously by immunization with rHBsAg DNA containing one or three copies of tumor epitope were both totally protected against further tumor challenge. These data suggest that enhancement of the effector response following immunization with rHBsAg DNA encoding multiple versus single copies of tumor antigen is not necessary for optimal and long-lived tumor protection. This is consistent with the view that tumor protection is brought about by central memory T cells (T_{CM}) stimulated by tumor challenge (Zanetti and Franchini, 2006).

Overall, the data confirm the efficacy of HBsAg DNA as a vector for the delivery of foreign CTL epitopes. They suggest that the magnitude of the CTL responses may be enhanced by provision of multiple copies of minigenes encoding a foreign CTL epitope, though in our system at least, the enhancement effect plateaus beyond provision of three copies. The data also indicate that increasing the size of the effector response by provision of multiple copies of a tumor epitope is not effective in increasing the levels of tumor protection, at least under the conditions of our experiments. While much of the antibody response to HBsAg is directed to determinant(s) in the external 'a' loop (see Fig. 1), and the CTL epitope replacements we report are located in transmembrane and external regions elsewhere, there is possibility that our recombinant vaccines would also elicit HBsAg antibody were appropriate conditions chosen for immunization (i.e. DNA injection into traumatized muscle and serology 6–12 weeks later) (Schirmbeck and Reimann, 2001).

While our data clearly indicate that rHBsAg DNA encoding three to five copies of the RAH minigene is more immunogenic than DNA encoding a single copy, factors in addition to, or other than, simple increase of copy number may contribute to this enhancement. Injected DNA encoding HBsAg DNA is likely to transfect a variety of cells, though only transfection of "professional" antigen-presenting cells (APCs) leads to activation of the CTL response via the intracellular (endogenous) processing pathway (Sbai et al., 2002). Following a single i.m. injection of HBsAg DNA, the antigen can be found in circulation for at least 1 month, suggesting sustained expression (Leclerc et al., 1997). Particle formation within the APC is not an essential requirement for this to occur (Sbai et al., 2002). Secretion of HBsAg particles (e.g. by DNA-transfected muscle cells) allows uptake of secreted particles by APCs for processing via the 'exogenous' pathway (Davis et al., 1993), thus providing a second mechanism for HBsAg CTL generation (Schirmbeck and Reimann, 2002). We have previously reported variable particle formation among rHBsAg DNAs encoding inserted foreign CTL epitopes (Woo et al., 2006). In short, particle formation, plus a range of other variables (i.e. variable antigen expression, stability of the HBsAg DNA and the expressed rHBsAg protein vivo, antigen processing, and

availability of T cell epitopes from the genetically engineered *HBsAg* gene) are all factors which might contribute to the differences in immunogenicity we report among the constructs used in the present study.

As well as having generic implication for the construction of chimeric HBsAg vaccines, our results also have specific implications for the development of a therapeutic vaccine for HPV-associated squamous carcinomas. Cervical carcinoma is recognized by the World Health Organization as being 100% attributable to HPV (Bosch et al., 2002), of which the 'high-risk' genotype HPV16 accounts for ca 60%. Cervical cancer is the leading cause of cancer-related death in women in developing countries, and causes ca 400,000 deaths per annum worldwide. While recently introduced prophylactic vaccines to prevent HPV infection are likely to decrease cervical cancer incidence, ca. 5 million women are predicted to die during the 10–20-year period until the effect of the vaccine is felt (Frazer, 2004). Uncontrolled expression of the HPV 16 E7 protein transforms cervical keratinocytes in a multi-step process which requires the persistence of the E7 oncoprotein in order for the cells to remains transformed. The E7 protein thus functions as a tumor-specific antigen to which immunotherapeutic strategies have been directed both in animal models of HPV associated tumor development, and in human clinical trials of therapeutic vaccines for cervical cancer (Fiander et al., 2006; Borysiewicz et al., 1996; Frazer et al., 2004). E7-directed protection against the HPV16 E7-expressing murine tumor TC-1 is provided by a $CD8^+$ CTL response to the RAH epitope used in the present study. This animal tumor model provides a surrogate for vaccine-induced CTL protection against E7-expressing cervical carcinoma in women, and for other HPV16 associated squamous cell cancers. A putative therapeutic vaccine for cervical cancer requires a powerful antigen delivery system to compensate for the suboptimal antigen presentation at the tumor site, and the local immunosuppressive milieu of the cervix (Tindle, 2002). Approaches taken so far of immunizing with peptides, whole proteins in recombinant viral vector, or as naked DNA to treat cervical cancer have produced disappointing results in clinical trials (Tindle, 2002). Such approaches have been limited by the availability of suitable adjuvants predisposing to a Th_1 response and licensed for use in humans, by pre-existing immunity to vector abrogating E7-directed responses, or by regulatory issues surrounding live vectors. Our present study suggests the application of HBsAg, already licensed as a human vaccine, for the delivery of E7-directed CTL responses for therapy of HPV16 associated cancers.

In summary, we demonstrate the efficacy of the replacement of DNA encoding endogenous CTL epitopes with DNA encoding a foreign tumor epitope at multiple sites within the *HBsAg* gene to generate tumor protective rHBsAg DNA vaccines. We show that while encoding three or more copies of the tumor epitope enhances CTL induction, it does not serve to augment tumor protection. There are clear implications for strategies for constructing recombinant HBsAg vaccines. We consider the use of rHBsAg vaccines in the context of a putative therapeutic vaccine for HPV-associated cervical and other cancers.

Materials and methods

Cloning procedures

The plasmid pcD3-HBsAgS (ayw subtype) (Netter et al., 2001) was engineered to delete the HBsAg-specific CTL epitopes VLQ, IPQ, FLG, GLS and SIL (Table 1), and to introduce the restriction enzyme sites *Bsi*W1, *Nhe*I, *Bsp*E1, *Blp*I and *Sac*II respectively by PCR-driven site-directed mutagenesis. Synthetic oligonucleotides encoding the RAH epitope were inserted into HBsAg through systematic sub-cloning into these restriction sites to create a series of constructs (Figs. 2a and 3a). Due to reading frame shifts caused by addition of restriction sites, codons for alanine and leucine (AA or A and AL) were included in the insert sequences at the 5′ and 3′ ends of the RAH oligomers. Standard molecular cloning and plasmid purification procedures were used. Positive clones were expanded using Wizard Plus SV minipreps DNA purification system (Progen, Australia) or EndoFree Plasmid Giga Kit (Qiagen, Australia) for DNA immunization. Sequences of constructs were verified via Terminator Sequencing (ABI) Big Dye 3.1.

Mice

$A2.1K^b$ mice make CTL responses restricted through $H\text{-}2^b$ class 1 molecules. Mice were housed under specific pathogen-free conditions, and used at 7–15 weeks of age. Experiments were conducted in accord with institutional animal ethics guidelines.

Peptides

Peptides were synthesised using F-moc chemistry and analysed by HPLC and by amino acid analysis by Chiron Mimotopes (Melbourne, Australia).

Immunization and restimulation of splenocytes

Mice were immunized twice at a 2-weekly intervals intradermally (i.d.) in the ear with 100 μg of purified plasmid DNA. 2 weeks later, spleens were removed and either the effector T response was quantified in ELISPOT assay *ex vivo*, or the splenocytes were restimulated *in vitro* for 6 days as described (Doan et al., 1998) with 1 μg/ml cognate peptide, and memory T cell response quantified by ELISPOT assay and/or ^{51}Chromium release assay. For peptide immunizations, mice were immunized subcutaneously (s.c.) at the tail base with 50 μg peptide + 0.25 μg tetanus toxoid (TT) as a source of T-helper epitopes + 10 μg Quil A adjuvant. 10 days later, spleens were harvested and splenocytes were restimulated as above.

Cells

EL4.A2 cells (Doan et al., 2005) and EL4.E7 cells are susceptible to specific CTL lysis through the $H\text{-}2^b$ restriction pathway. EL4.E7 cells were derived by transfection of EL4 cells

with the pJ4ΩE7 expression plasmid as described (Tindle et al., 1995). TC-1 is a tumor cell line derived from primary lung epithelial cells of C57BL/6 (H-2^b) mice by co-transfection with *HPV16 E6*, *E7* and *ca-Ha ras* oncogenes (Lin et al., 1996). Cells were maintained as described (Doan et al., 1998). TC-1 cells were prepared for injection in Hank's balanced salt solution.

Murine IFN-γ ELISPOT assay

Epitope-specific gamma interferon (IFN-g)-secreting spleen cells were enumerated *ex vivo* by an enzyme-linked immunospot (ELISPOT) assay with minimal $CD8^+$ T-cell epitope peptides, essentially as described (Wiethe et al., 2003). IFN-g spots were counted using an AID EliSpot reader system. Results were calculated as IFN-γ-positive cells/10^6 spleen cells. Experimental values were compared for significant difference using chi-square analysis.

^{51}Chromium release cytotoxicity assay

Assays were conducted as previously described (Doan et al., 1998). In summary, target cells (10^4 per well) sensitised at 37 °C for 1 h with 1 μg/ml cognate or irrelevant peptide, or medium alone, and labelled with 100 μCi ^{51}Chromium (Cr), were incubated with effector cells at various effector: target cell ratios in triplicate in 96-well microtitre plates. Negative controls included wells containing target cells but no effector cells (='background'). Supernatants were harvested from CTL assays at 4 h, and ^{51}Cr release quantified by gamma counting. Results are expressed as percent cytotoxicity ± standard deviation (^{51}Cr release in experimental wells − background/detergent-mediated total release − background) × 100%.

Tumor prevention

Groups of mice were immunized with 100 μg plasmid DNA id, or 100 μg of E7 peptide + tetanus toxoid + Quil A sc. TC-1 cells, which express the E7 tumor-associated antigen of human papillomavirus type 16, were subsequently injected (2×10^5 in 0.1 ml Hank's buffered salt solution) s.c. on the flank. (The tumor dose was predetermined by titration experiments to discern a minimal dose, giving rise to tumor in 80–100% of unimmunized mice.) Tumor growth was monitored at intervals, and mice were euthanized when tumor volume exceeded 500 mm^3. Unimmunized or HBsAg wild type (W/T) immunized mice received the same number of TC-1 cells and served as a control. Results are presented as Kaplan–Meier curves of % surviving mice, and treatment groups were compared using the Log Rank statistic.

Tumor therapy

Non-palpable tumor

Groups of mice were injected with 5×10^4 TC-1 cells subcutaneously on the flank. At 3 days later, the mice were immunized three times at 4-day intervals, and subsequent tumor growth quantified as number of tumor-bearing mice and as tumor volume. Tumor volume was derived from caliper measurement in two perpendicular dimensions by the formula S^2L, where S is the shorter dimension and L is the longer dimension of the tumor. Tumor volumes between groups were compared by analysis of variance (ANOVA).

Palpable tumor

Groups of mice were injected with TC-1 cells as above. When developing tumors became palpable ca. 14 days later with approximate volumes of 80 mm^3, mice were immunized 3 times at 3-day intervals, and thereafter tumor volume was monitored every 3–4 days. Tumor volumes were measured as above. Results are presented as Kaplan–Meier curves of % tumor-free mice, and treatment groups were compared using the Log Rank statistic.

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