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Codon Modified Human Papillomavirus Type 16 E7 DNA Vaccine Enhances Cytotoxic T-Lymphocyte Induction and Anti-tumour Activity

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Polynucleotide immunisation with the E7 gene of human papillomavirus (HPV) type 16 induces only moderate levels of immune response, which may in part be due to limitation in E7 gene expression influenced by biased HPV codon usage. Here we compare for expression and immunogenicity polynucleotide expression plasmids encoding wild-type (pWE7) or synthetic codon optimised (pHE7) HPV16 E7 DNA. Cos-1 cells transfected with pHE7 expressed higher levels of E7 protein than similar cells transfected with pW7. C57BL/6 mice and F1 (C57× FVB) E7 transgenic mice immunised intradermally with E7 plasmids produced high levels of anti-E7 antibody. pHE7 induced a significantly stronger E7-specific cytotoxic T-lymphocyte response than pWE7 and 100% tumour protection in C57BL/6 mice, but neither vaccine induced CTL in partially E7 tolerant K14E7 transgenic mice. The data indicate that immunogenicity of an E7 polynucleotide vaccine can be enhanced by codon modification. However, this may be insufficient for priming E7 responses in animals with split tolerance to E7 as a consequence of expression of E7 in somatic cells.

INTRODUCTION

Infection with human papillomavirus (HPV), and particularly with genotypes 16, 18, 31, or 35, is a major antecedent of anogenital malignancy (Bosch *et al.,* 1995; Wallin *et al.,* 1999). The E7 protein of HPV16 is expressed constantly in cervical cancer cells (Seedorf *et al.,* 1987) and contributes to the transformation and maintenance of malignant phenotype in epithelial cells *in vitro* (Münger, 1995). Mouse and human T- and B-cell epitopes of the E7 protein have been identified (Tindle *et al.,* 1990, 1991; Feltkamp *et al.,* 1993; Ressing *et al.,* 1995; Altmann *et al.,* 1992) and immunity to E7 has been proposed as a means to prevent or treat HPV-associated cervical malignancies.

Polynucleotide vaccines can prevent a variety of infections in animals (Benton and Kennedy, 1998; Donnelly *et al.,* 1997; Pardoll and Beckerleg, 1995). Immunogenicity of HPV16 E7 DNA has been demonstrated following intramuscular or intradermal gene delivery (Chen *et al.,* 1999; Han *et al.,* 1999; Shi *et al.,* 1999). However, the cytotoxic T-cell response to E7 polynucleotide vaccines is not strong (Michel *et al.,* 2002), and several strategies have been applied to increase the potency of E7 polynucleotide vaccines. These include targeting of antigens into antigen processing and presentation pathways by fusion to genes encoding proteins enhancing protein spreading between cells (Hung *et al.,* 2001a), ligands for APC proliferation receptors (Hung *et al.,* 2001c), for antigen receptors on APC (Hung *et al.,* 2001b), for heat shock protein (Chen *et al.,* 2000b), for lysosomal-associated membrane protein (Ji *et al.,* 1999), and for proteins with no obvious immune function (Cheng *et al.,* 2001). Alternatively, mutations in E7 which improve expression (Shi *et al.,* 1999) or prime boost strategies with other antigen delivery systems (Chen *et al.,* 2000a) have been used. Recently, the influence of codon usage on the immunogenicity of polynucleotide vaccines for viral infection has been recognised (Andre *et al.,* 1998; Vinner *et al.,* 1999; Zur Megede *et al.,* 2000; Deml *et al.,* 2001a; Leder *et al.,* 2001). PV capsid protein expression depends on the match between codon usage and tRNA availability in target cells (Zhou *et al.,* 1999), and codon modified HPV16 L1 and L2 HPV6bL1 and BPV1 L2 polynucleotide vaccines are more immunogenic than unmodified vaccines, when delivered by the gene gun (Liu *et al.,* 2001; Leder *et al.,* 2001). Here we investigated whether codon modification can enhance the immunogenicity of an HPV16 E7 polynucleotide vaccine by examining humoral and cellular immune responses and host protection against tumour challenge. We show that polynucleotide vaccination induces humoral immune responses to E7 in nontransgenic and partially E7 tolerant (Frazer *et al.,*

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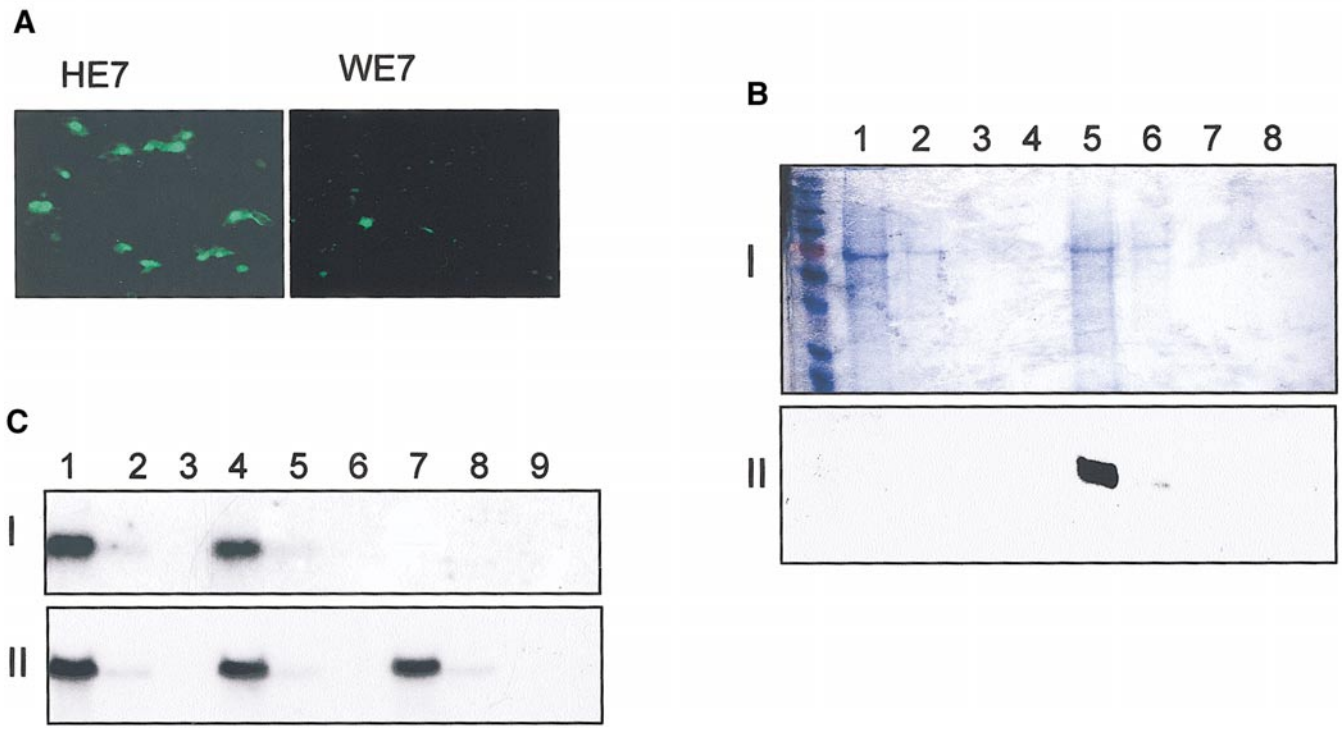

FIG. 1. Expression of E7 protein and of the corresponding mRNA from Wt or codon modified E7 genes. Cos-1 cells were transfected with wt or modified E7 plasmids. (A) Immunofluorescence analysis of Cos-1 cells transfected by pHE7 and pWE7 with anti-E7 antibodies at 48 h after transfection. E7 protein was visible in the cytoplasm of cells transfected by pHE7, whereas cells transfected by pWE7 sequences were occasionally positive (epifluorescence microscopy, original magnification ×100). (B) Immunoblot analysis of Cos-1 cell lysates transfected with pWE7 (lanes 1–4) and pHE7 (lane 5–8) at 48 h after transfection. Lanes 1–4 contain WE7 cellular lysates 10, 2, 0.4, and 0.08 μg protein, respectively. Lane 5–8 contain HE7 cell lysates 10, 2, 0.4, and 0.08μg protein, respectively. (I) Coomassie-blue-stained protein gel—markers (M) are 10, 15, 20, 25, 40, 50, 60, 85, 120, and 190 kDa. (II) Immunoblot probed with E7-specific MAb—reactive band is ~16 kDa. (C) Northern blot analysis of total RNA isolated from Cos-1 cells transfected by pHE7 (lanes 1–3) or pWE7 (lanes 4–6) or mock transfected (lanes 7–9) at 48 h after transfection. Total cellular RNA was extracted from whole-cell preparations of cells transfected with pWE7 or pHE7. Northern blots were probed with a mixture of ^{32}P-labelled WE7 and HE7 sequences (I) or with β-actin probe (II). Lanes 1–3 contain pHE7 total cellular RNA 10, 1, and 0.1 μg, respectively. Lanes 3–6 contain pWE7 total cellular RNA 10, 1, and 0.1 μg, respectively, and lanes 7–9 contain normal cellular RNA, 10, 1, and 0.1 μg, respectively.

1998) transgenic mice and that codon modification of an E7 polynucleotide vaccine toward human consensus codon usage significantly increases E7-specific CTL responses, but only in the nontransgenic mice.

RESULTS

Codon modified E7 gene significantly increased E7 expression

To compare the expression of codon modified (pHE7) and wild-type (pWE7) HPV16 E7 genes, Cos-1 cells were transfected with pWE7 and pHE7. E7 protein was determined 48 h after transfection by immunofluorescence and immunoblot, and E7 mRNA was determined by Northern blot. Data (Fig. 1) are representative of at least three experiments for each transfection. Of cells transfected with pHE7, 30–40% produced E7 protein within the cell cytoplasm. Only occasional cells transfected with pWE7 produced sufficient E7 protein to be recognized by immunofluorescence (Fig. 1A). E7 was in each case detected in the cytoplasm of transfected cells, which was unexpected as E7 is thought to be a nuclear protein (Greenfield *et al.,* 1991). E7 protein expression was also assessed by immunoblot analysis. Immunoreactive E7 protein was detected in lysates of cells transfected with pHE7 but not pWE7 (Fig. 1B). To establish whether the observed differences in E7 protein expression for pWE7 and pHE7 reflected differing transcription or translation of the E7 gene, total cellular RNAs were prepared from Cos-1 cells transfected 48 h previously with the E7 expression plasmids. Using β-actin as an internal standard, transcription of the codon modified and wild-type E7 plasmids was quantified and the ratio of E7 mRNA (pWE7:pHE7), normalised to the β-actin internal standard, was 1:1.3 (Fig. 1C). Higher expression of E7 protein with the codon modified E7 plasmid was thus a consequence of increased E7 mRNA translation, rather than increased E7 gene transcription or increased E7 mRNA stability.

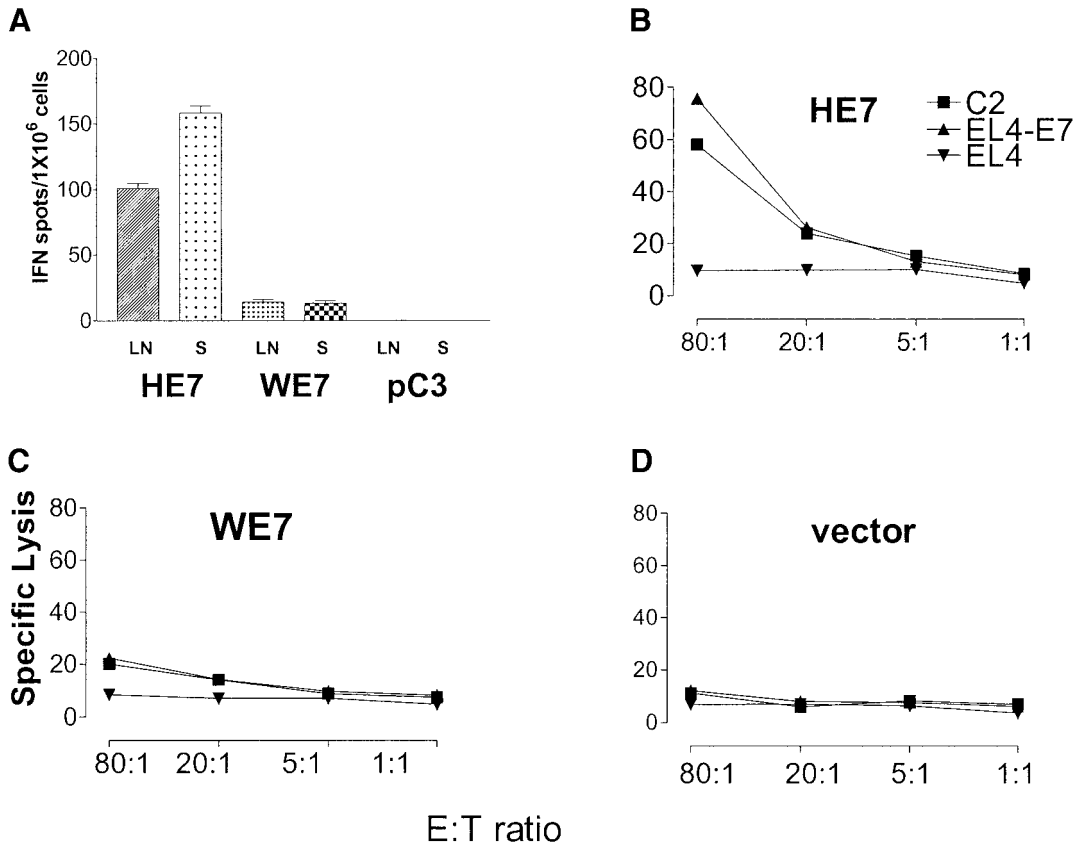

FIG. 2. Assay of E7-specific T cells from C57Bl/6 mice immunised with HPV16 E7 DNA vaccines pWE7 and pHE7. (A) Mice were immunised at days 0 and 14 with codon modified (hE7) or wild-type E7 (wE7) plasmids or with pCDNA3 (pC3) as shown. Splenocytes (S) and lymphocytes (LN) were harvested on day 24. The number of IFN-γ-producing E7-specific $CD8^+$ T-cell precursors was determined using the ELISPOT assay. The spot numbers were the mean of triplicate determinations ± 1 SE in each vaccinated group. (B–D) Splenocytes were isolated from mice immunised with codon modified (B) or wild-type (C) E7 or with control plasmid (D) and were stimulated *in vitro* with HPV16 E7 peptide (amino acids 49–57). The generated effector cells were used in standard ^{51}Cr release assays against E7 peptide-coated (EL4-E7) or uncoated (EL4) target cells and against a cell line expressing endogenous E7 (C2).

Increased immunogenicity of HPV16 E7 DNA with codon modification

To examine *in vivo* immune responses induced by the wild-type and codon modified E7 expression vectors, female C57 BL/6 mice were immunised intradermally into the abdominal skin using the Helios Gene Gun (Bio-Rad Laboratories). Two weeks after the second immunisation, spleen cells and lymph cells were isolated from each mouse (four mice per group). To quantify IFN-γ secreting E7-specific CD8 T-cell precursor frequencies generated by E7 DNA vaccines, the ELISPOT assay for the H-2 D^b restricted E7 T-cell epitope RAHYNIVTF was used. As shown (Fig. 2A), 120 to 160 IFN-γ spot-forming T cells specific for RAHYNIVTF were detected per 10^6 lymphocytes or splenocytes derived from pHE7 immunised mice, compared to 15 to 20 IFN-γ spot-forming CD8+ T cells/10^6 splenocytes or lymphocytes from pWE7 vaccinated mice. No peptide-specific IFN-γ spot-forming CD8+ T cells were observed in pcDNA3 immunised mice.

CD8+ T-cell cytolytic precursors generated by polynucleotide immunisation were determined by standard chromium release cytolytic assay (Fernando *et al.,* 1998a). Bulk effector CTL from C57BL/6 mice immunised by pHE7 DNA vaccine specifically lysed 60–80% of HPV16 E7 peptide-coated EL4 and E7 transfected tumour target cells (C2) (Fig. 2B), but did not lyse EL-4 cells in the absence of E7 peptide, confirming the specificity of the CTL responses. Bulk effector CTL from C57BL/6 mice immunised by WE7 DNA vaccine specifically lysed about 20% of HPV16 E7 peptide-coated EL4 and E7 transfected tumour target cells (C2) (Fig. 2C), compared with no lysis of these targets by CTL derived from vector DNA immunised mice (Fig. 2D).

Sera from mice immunised with each E7 polynucleotide vaccine were tested in ELISA using nine overlapping E7 peptides as substrate (Frazer *et al.,* 1995b). Peptide-specific antibody was detected in E7 polynucleotide vaccinated C57BL/6 mice (Fig. 3) and there was no statistically significant difference in the response to any

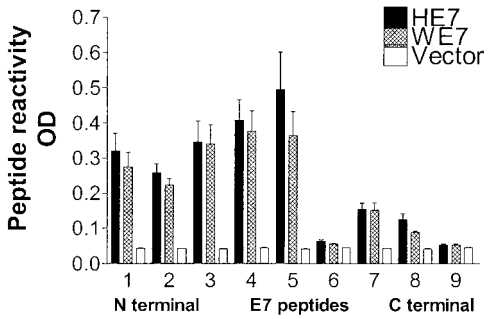

FIG. 3. E7-specific antibody responses from C57Bl/6 mice immunised with E7 polynucleotide vaccine. Mice (10 per group) were immunised with control vector (vector) or HPV16 E7 DNA vaccines (WE7 and HE7) via a gene gun. Serum samples were obtained from immunised mice 14 days after the second vaccination. E7-specific antibody was detected by nine overlapping 18- to 22-mer peptides, GF101–GF109 (designated 1–9); scanning the length of the E7 protein. ELISA was carried out using serial dilutions of sera. The results from the 1:100 dilution are presented and show the mean absorbency (OD 490 nm) ± 1 SE. Standard errors shown represent variability from mouse to mouse across the group.

E7 peptide between the pHE7 and pWE7 vaccinated groups by unpaired *t* test. E7-specific antibody reactivity against peptides corresponding to the E7 C terminus was lower than for peptides corresponding to E7 N-terminus, as has previously been observed for immunisation with E7 protein (Fernando *et al.,* 1998b).

HPV16 E7 vaccine protected mice against the growth of TC-1 tumours

We next determined whether E7 polynucleotide immunisation could induce protective immunity against challenge with an HPV16 E7 expressing tumour. C57Bl/6 mice (eight per group) were immunised twice with pHE7 or pWE7 polynucleotide vaccine and challenged subcutaneously with 2×10^6 TC-1 tumour cells 14 days later. This dose of tumour cells is the minimal dose to induce tumours in 100% of inoculated mice, based on our previous experiments. For the pHE7 vaccinated mice, 100% mice remained tumour-free at 14 and 30 days after the TC-1 cell challenge, whereas 50% of mice receiving pWE7 DNA vaccination had developed small tumours by day 14. Mice receiving a control plasmid each developed large tumours by 14 days after tumour challenge (Fig. 4). Codon modified E7 DNA could thus significantly enhance E7-specific CTL and induce better immunity against the growth of TC-1 cells, when compared to wild-type E7 DNA vaccination.

E7 DNA vaccine induced antibody but not CTL responses in E7 transgenic mice

Previously, we have shown that specific immunisation of E7 transgenic mice with E7 protein failed to induce an E7-specific cellular response or to prevent or control E7-associated tumour development, in contrast to non-E7 transgenic control mice (Frazer *et al.,* 1995a). Immunisation with E7 peptide-pulsed DCs has been reported to overcome E7 directed CTL tolerance (Doan *et al.,* 2000). Dendritic cells can be directly transfected by gene-gun-mediated gene delivery. We therefore determined whether E7 DNA vaccination via a gene gun could induce a cellular immune response in E7 transgenic mice. As with C57 BL/6 mice, E7 peptide-specific antibody was detected in E7 polynucleotide vaccinated F1 (FVB× C57BL/6) mice transgenic for HPV16E7 (Fig. 5A), confirming previous findings with E7 protein immunisation that the Th- and B-cell compartments are not tolerised to E7 in these mice. However, no E7-specific IFN-γ secretion by CD8 T cells was detected in ELISPOT assays following either vaccine (Fig. 5B). These data suggest that the E7 CTL tolerance in E7 transgenic mice cannot be overcome by priming of skin-derived DCs.

DISCUSSION

Our data confirm that a polynucleotide vaccine encoding E7 protein can induce an E7-specific CTL response and some tumour protection *in vivo* and demonstrate that the CTL response can be markedly enhanced by codon modification which results in improved translation of E7 mRNA. As found by others for HIV proteins (Haas *et al.,* 1996) and ourselves for HPV L1 proteins (Zhou *et al.,* 1999), altering codon usage in an E7 polynucleotide vaccine toward mammalian consensus usage results in better expression of protein *in vitro* and an improved CTL response and tumour protection *in vivo.* After gene gun immunisation, directly transfected dendritic cells may prime CD8+ cells directly (Porgador *et al.,* 1998) or CD8+ cells may be induced by cross priming (Cho *et al.,* 2001). The gene transfer procedure results in the activa-

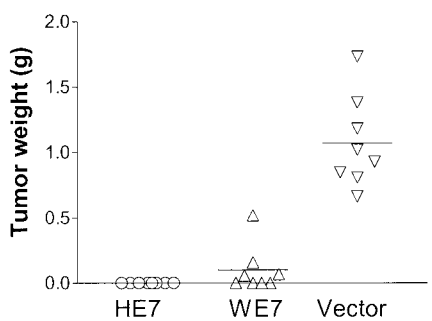

FIG. 4. Tumour weight in mice immunised with control vector (vector) or HPV16 E7 DNA vaccines WE7 and HE7. Mice were immunised at days 1 and 14 and at day 24 they were challenged with 2×10^6 TC-1 cells subcutaneously. The data represent the weight of individual tumours measured at the end of week 2 after tumour challenge. Bars show the mean tumour weight. The tumour weight from the mice immunised with HE7 is significantly ($P = 0.01$) less than that from the mice immunised with WE7.

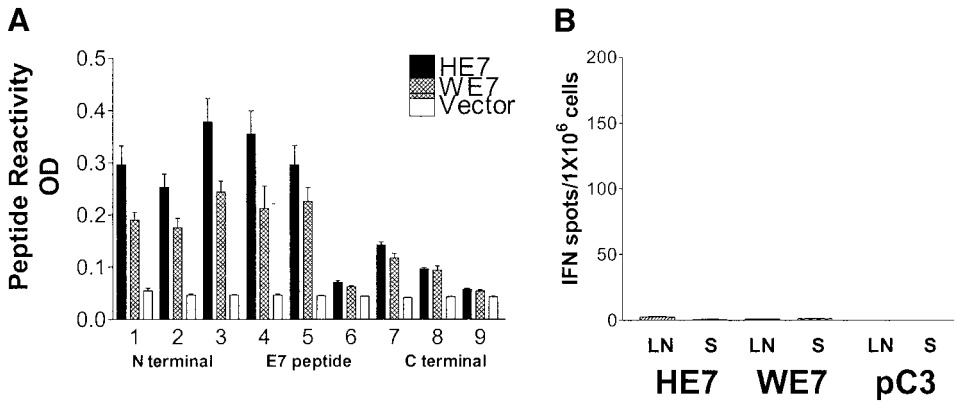

FIG. 5. Immune response of K14E7 transgenic mice to immunisation with codon modified or wild-type E7 polynucleotide vaccine. (A) Antibody to E7 peptides was measured as in Fig. 3. (B) E7-specific CD8 T cells were measured by ELISPOT as for Fig. 2A.

tion of DCs and initiates migration to regional lymph nodes, where antigen-expressing DCs efficiently stimulate proliferation of antigen-specific CD8+ as well as CD4+ T lymphocytes (Tuting *et al.,* 1999). *In vivo* administration of DC that have been transfected *in vitro* is sufficient to induce both humoral and cellular immune responses (Doan *et al.,* 2000; Manickan *et al.,* 1997; Timares *et al.,* 1998). While the most likely explanation for better immune responses to a polynucleotide vaccine following codon modification is better protein expression, as the amount of protein delivered is directly linked to the magnitude of the induced immune response (Wherry *et al.,* 1999), it has also been shown that immunostimulatory CpG motifs can stimulate IL-12 release and hence promote the generation of CTL by polynucleotide vaccines (Klinman *et al.,* 1998; Sato *et al.,* 1996). However, codon modification of the E7 gene did not increase the number of GACGTT or AACGTT typical CpG immunostimulatory sequences within the modified gene (data not shown), and a similar antibody response was observed to E7 whether the E7 gene was codon modified or not. Thus, codon modification of E7, which is associated with higher levels of E7 expression, seems specifically to enhance the CTL response to E7, perhaps because higher levels of antigen expression are required to achieve robust T-cell-mediated immunity than a measurable antibody response.

The current observation contrasts with recent reports that codon modification significantly increased antibody responses for other genes (Andre *et al.,* 1998; Vinner *et al.,* 1999; Zur Megede *et al.,* 2000; Deml *et al.,* 2001a; Leder *et al.,* 2001). The mechanism of induction of antibody response by polynucleotide vaccines is unclear. Where intracellular antigens are poorly secreted, as is the case for E7 protein, induction of antibodies may be due to the lysis of the expressing cell by the specifically induced cellular immune response (Griesenbach *et al.,* 1998). The various early ORFs of PV are nuclear proteins which induce host protective cell-mediated immunity, but not a humoral response when administered to rabbits. Rabbits immunised with CRPV E6-DNA developed E6-specific cellular immunity as determined by proliferation assays but did not develop detectable humoral immunity to E6 proteins, as evaluated by ELISA using two different E6 antigen preparations (Sundaram *et al.,* 1998). Intramuscular injection of plasmid DNA encoding CRPV E1, E2, E6, or E7 induced CD4+ T-cell-mediated but not humoral immune responses and did not result in the protection of rabbits from virus infection (Han *et al.,* 1999). Gene-gun-based intracutaneous vaccination with a combination of papillomavirus E1 and E2 genes induced strong protective antivirus immunity but not humoral immune response (Han *et al.,* 2000). Previously we reported that E7 protein degrades very quickly in transfected cells (Park *et al.,* 1993). The results in this paper were further confirmed by the observation that the codon optimised E7 protein can be better detected in the presence of a mixture of proteasome inhibitors. Thus, rapid intracellular degradation of excess E7 protein might explain the equal E7 antibody response after pHE7 and pWE7 DNA vaccination, which presumably reflects the minimal amount of intact E7 bound by B cells primed by T helper responses in the draining lymph node and a need for greater levels of E7 expression for optimal CTL responses than for optimal antibody response.

The K14E7 transgenic mice (Lambert *et al.,* 1993) express E7 in the skin and thymus and have been evaluated immunologically as a potential model in which to test immunotherapy for papillomavirus-associated epithelial cancer. In a previous study, we showed that these E7 transgenic mice demonstrate split tolerance to E7 protein, in that specific immunisation of E7 transgenic mice with E7 protein induced antibody and T helper cells specific for E7 but failed to induce E7-specific cytotoxic T cell responses sufficient to prevent or control E7-associated tumour development (Frazer *et al.,* 1998). It has been reported that administration of a given peptide in adjuvant to the immune system may induce tolerance,

whereas administration of the same peptide on DCs is stimulatory (Toes *et al.,* 1998). Peripheral tolerance to HPV16 E7 oncoprotein can occur by cross-tolerisation, and immunisation with E7 peptide-pulsed DCs overcame E7-directed CTL tolerance (Doan *et al.,* 2000). However, we failed to induce E7-specific CTL responses in K14 E7 F1 (FVB× C57 BL/6) E7 transgenic mice via a gene-gun-mediated polynucleotide vaccination. Why presentation of E7-specific epitope by peptide-pulsed DCs induces CTL activation, whereas polynucleotide vaccine primed skin derived DCs could not induce CTL activation, is unclear. One explanation might be that only a very small number of skin-derived DCs were transfected by E7 genes, whereas the previous study used a large number of peptide-pulsed DCs for immunisation. Alternatively, polynucleotide vaccination might not activate skin-associated LC as effectively as *in vitro* manipulation of bone-marrow-derived DC.

Other polynucleotide vaccine strategies have been used effectively to enhance E7-directed CTLs. The E7 gene, modified so that the open reading frame encoding mutations in two zinc-binding motifs or mutagenesis of the pRB-binding site, demonstrated a significant enhancement of immunogenicity and better tumour protection in mice than did a wild-type E7 DNA vaccine (Shi *et al.,* 1999; Smahel *et al.,* 2001). When administered as a polynucleotide vaccine, E7 linked to the sorting signal of the lysosome-associated membrane protein-1 (Sig/E7/LAMP-1) generated stronger E7-specific CTL activity, higher numbers of E7-specific CD8+ cell precursors, and higher titres of E7-specific antibody than E7 alone. Both E7 DNA and Sig/E7/LAMP-1 DNA generated potent anti-tumour immunity in the liver and lung metastases models (Chen *et al.,* 1999; Ji *et al.,* 1999), suggesting that these models require less CTL activity for tumour protection than the primary tumour model used in the current study.

Two nonconservative nucleotide substitutions were detected in the codon modified E7 gene used in the current studies. These mutations are probably nonsignificant for protein immunogenicity, as they do not alter any of the known H-2^b restricted T or B epitopes of HPV16 E7 (Altmann *et al.,* 1992; Comerford *et al.,* 1991; Tindle *et al.,* 1990; Tindle *et al.,* 1991; Feltkamp *et al.,* 1993). The alteration in the primary DNA sequence might potentially increase E7 mRNA stability or remove mRNA instability elements which could increase the E7 mRNA pool and hence the rate of E7 expression. However, the comparable E7 mRNA levels observed *in vitro* for the codon modified and unmodified E7 suggest that this is unlikely. Alternatively, the amino acid changes resulting from the two nonconservative nucleotide substitutions might increase the stability of the E7 protein, to account for the higher steady-state levels of E7 observed *in vitro,* as native sequence E7 is known to have short half-life *in vitro* and *in vivo* (Park *et al.,* 1993; Selvey *et al.,* 1994). However, the enhanced CTL immune response to the codon modified E7 after delivery as a polynucleotide vaccine *in vivo,* when compared with the unmodified E7, should reflect a larger pool of proteasome degraded E7, rather than a reduced turnover of E7 as a consequence of the two amino acid substitutions, as protein modifications including ubiquitination (Boyle *et al.,* 1998; Tobery and Siliciano, 1997) which enhance protein degradation also enhance CTL responses to the modified proteins. Alternatively, the modifications to the E7 primary sequence might alter some of the capacity of E7 to interfere with its own presentation. However, the mutants observed are not recognised as loss of function mutants in a protein extensively studied for functional domains, and expression of the modified E7 protein *in vitro* results in cell death, as is observed for wild-type E7 (data not shown). Thus, codon modification has most likely enhanced the efficiency of translation of E7 mRNA, accounting for the increased protein levels observed and hence increased immunogenicity, as has been observed for a range of other genes *in vitro* and *in vivo* (Deml *et al.,* 2001b; Koide *et al.,* 2000; Narum *et al.,* 2001; Stratford *et al.,* 2000; Uchijima *et al.,* 1998).

In conclusion, codon optimisation can enhance the CTL response induced by E7 polynucleotide vaccination sufficiently to protect mice against tumour growth. Synthetic genes with optimised codon usage may thus represent an effective strategy to increase the efficiency of polynucleotide vaccines targeting tumour antigens.

MATERIALS AND METHODS

Mice and cell lines

Specific pathogen-free female C57BL/6 and F1 (C57× FVB) K14E7 transgenic mice (Herber *et al.,* 1996) aged 6 to 8 weeks were purchased from the Animal Resource Centre (Australia) and maintained under clean conditions in a conventional mouse house. The HPV16 E7 transfected EL4 cell line, C2 (Tindle *et al.,* 1995), and the parent cell line (EL-4) were maintained in complete RPMI 1640 medium plus 10% fetal bovine serum (CSL, Australia).

Construction of codon modified HPV16 E7 DNA vaccines

The HPV16 E7 gene was modified to substitute mammalian preferred codons for rarely used codons (Zhou *et al.,* 1999). A synthetic E7 gene, incorporating a consensus Kozak sequence and flanked by appropriate restriction enzyme sites, was assembled using overlapping long synthetic oligonucleotides amplified by PCR. Four primers were purchased from Life Technologies:

Primer A: 5′ccgggtaccgccgccaccatgcacggcgacacccccaccctgcacgagtacatgctggacctgcagcccgagaccaccgacctctactgctac3′;

Primer B: 5′tagtgggcgcggtcgggctcggcctggccggcggggccgtcgatctcgtcctcctcctcgctgctgtcgttgagctgctcgtagcagtacaggtcggtg3′;

Primer C: 5′gagcccgaccgcgcccactacaacatcgtgaccttctgctgcaagtgcgacagcaccctgcgcctatgcgtgcagagcacccacgtggacatccgcaccc3′;

Primer D: 5′cgcgaattcttagggcttctgggagcagatggggcacacgatgcccagggtgcccatcagcaggtcctccagggtgcggatgtccacgtgggtg3′.

A 5′ primer ccgggtaccgccgccaccatgcatggagatacacctaca and a 3′ primer cgcgaattcttatggtttctgagaacagat were used to amplify the unmodified E7 gene, incorporating a Kozak sequence and flanked by restriction sites, from plasmid pHPV16 (Park *et al.,* 1993). The unmodified and codon modified E7 genes were cloned into the *Kpn*I and *Eco*RI sites of the pcDNA3 mammalian expression vector (Invitrogen), giving expression plasmids pWE7 and pHE7. Recombinant clones were sequenced to confirm the orientation and integrity of the inserted E7 sequences. The codon modified recombinant clones demonstrated two point mutations in the primary nucleotide sequence resulting in a leucine to phenylalanine substitution at aa 28 and a glutamine to arginine substitution at aa 70.

Immunofluorescence and immunoblot staining

For immunofluorescence staining, Cos-1 cells grown on eight-well chamber slides were transfected with 0.5 μg E7 expression plasmid using lipofectamine (Gibco). Twenty-four hours after transfection, a mixture of proteasome inhibitors ALLN (Sigma, 20 μg/ml), leupeptin (Sigma 100 μm/ml), and cycloheximide (Sigma, 5 μg/ml) was added and maintained at this concentration for a further 24 h. Forty-eight hours after transfection, cells were fixed and permeabilized with 85% ethanol. The slides were blocked with 5% milk–PBS and probed with E7-specific monoclonal antibody 6D (Tindle *et al.,* 1990) followed by fluorescein-isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) (Sigma). For immunoblotting assays, cells were harvested 48 h after transfection and lysed in sodium dodecyl sulphate (SDS) loading buffer. The cellular proteins were separated by SDS–polyacrylamide gel electrophoresis in a 10% gel and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS and probed with anti-E7 monoclonal antibody at a dilution of 1:2000. Bound antibody was detected by incubation of the membrane with horseradish-peroxidase-conjugated sheep anti-mouse antibody (Silenus, Australia) at a dilution of 1:1000 and visualized using enhanced chemiluminescence (Amersham).

Northern blotting

Total RNA was extracted from cells transfected 48 h previously with E7 expression plasmids using lipofectamine (Gibco). Total cellular RNA was separated on a 1.5% agarose gel in the presence of formamide. The RNAs were blotted onto a nylon membrane and E7 mRNAs were analysed by Northern blot with ^{32}P-labelled 1:1 mixed wt E7 and HE7 cDNA probes and a β-actin cDNA, respectively.

DNA immunisation

Plasmids were purified using a Qiagen Plasmid Mega kit (Qiagen, Chatsworth, CA) and dissolved in PBS at a concentration of 1 μg/μl. Female C57 BL/6 mice and F1 (C57× FVB) E7 transgenic mice, 6 to 8 weeks old, were immunised by particle bombardment with DNA-coated gold beads (2 μg DNA/dose) using the helium-powered Helios Gene Gun delivery system (Bio-Rad Laboratories, Richmond, CA). A total of 1.0 μg of DNA was coupled to 0.5 mg of 1.0-μm-diameter gold particles, as recommended by the manufacturer. DNA-coated microcarriers were delivered into the abdominal epidermis at a helium pressure setting of 400 psi.

Peptide ELISA

Nine peptides 22 amino acids long, overlapping by 12 amino acids, spanning the full length of HPV16 E7 (Frazer *et al.,* 1995) and synthesized by Chiron (Melbourne, Australia), were used to determine E7 antibody response. Microtiter plates (Dynatech) were coated overnight with 50 μl of different E7 peptide. After being blocked for 2 h at 37°C with 100 μl of 5% milk, 0.05% Tween 20 in PBS, plates were washed three times with PBS/0.05% Tween 20. A total of 50 μl of sera at dilution of 1:100 was added for 1 h. Plates were washed again and 50 μl of goat anti-mouse IgG peroxidase conjugate was added at a 1:5000 dilution. After 1 h, plates were washed before the addition of OPD substrate. Absorbance was measured after 20–30 min at 490 nm in a Dynatech automated plate reader. As a control, readings from wells coated with peptide were compared to wells coated with PBS only.

ELISPOT assay

The ELISPOT assay (Miyahira *et al.,* 1995) was modified to detect HPV16 E7-specific CD8+ T cells. Ninety-six-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml rat anti-mouse IFN-γ antibody (clone R4-6A2, PharMingen, San Diego, CA) in 50 μl of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. A total of 1×10^6 fresh isolated spleen and lymph node cells were added to the well

along with 20 IU/ml IL-2. Cells were incubated at 37°C for 24 h either with or without 1 μg/ml HPV16 E7-specific H-2D^b CTL epitope (RAHYNIVTF, single-letter amino acid code). After culture, the plate was washed and then incubated with 5 μg/ml biotinylated IFN-γ antibody (clone XMG1.2, PharMingen, Franklin Lakes, NJ) in 50 μl in PBS at 4°C overnight. After six washes times, 1.25 μg/ml peroxidase (Sigma) in 50 μl of PBS was added and incubated for 2 h at room temperature. After the washings, spots were developed by the addition of 50 μl of 5-bromo-4-chloro-3-3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Manheim, Indianapolis, IN) and incubated at room temperature for 1 h. The spots were counted using a dissecting microscope.

In vivo tumour protection

Mice were subcutaneously challenged in the scruff of the neck with 2×10^6 cells/mouse of TC-1 tumour cell (Lin *et al.,* 1996), kindly provided by Dr. T. C. Wu. Tumour weight was recorded as previously described (Fernando *et al.,* 1998a). Generally, in groups of mice where tumours were present at 14 days, all mice were killed and tumours dissected and weighed. Groups of mice without tumours at 14 days were observed to day 30 for tumour development.

Cytotoxic T-lymphocyte assays

Splenocytes were prepared from immunised animals and CTL activity was assessed after a 3-day *in vitro* restimulation with rIL-2 (Sigma) and peptide RAHYNIVTF as previously described (Frazer *et al.,* 1998). Assays were performed in triplicate, and spontaneous ^{51}Cr release from the various targets did not exceed 15%.

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