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Señores
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Re.: Código 02-01-444
Expediente No. 00-66.606
Solicitud de Patente
SmithKline Beecham
Biologicals s.a.

En relación con el expediente de la referencia y dando cumplimiento a la última providencia recaída sobre este asunto por medio del presente escrito me permito manifestarles lo siguiente:

1. De acuerdo con lo solicitado en el concepto técnico No. 1350 notificado mediante oficio No. 7998 me permito adjuntar los siguientes documentos:

   a.) El artículo de TINDLE ET AL. Virology, Volume 200, Issue 2. pag 547 a 557.

   b.) El artículo LONDOÑO ET AL. Vaccine, Volume 14, issue 6, pag. 545-552.

2. Con relación al concepto técnico No. 1347 notificado mediante oficio No. 7997 la sociedad solicitante desea efectuar los siguientes comentarios acerca de la novedad y el nivel inventivo de esta invención:

   a.) Adjuntamos un juego de las reivindicaciones modificadas las cuales son equivalentes a las permitidas en Europa. En estas reivindicaciones, se ha indicado la naturaleza del antígeno HPV. Como tales, las reivindicaciones son novedosas sobre la técnica anterior.

b.) Con respecto al nivel inventivo, se puede sostener que la persona versada en la técnica no tiene ninguna motivación para combinar la referencia de la técnica anterior tal como lo señala el Examinador.

3. Londoño y otros no hacen referencia al uso de adyuvantes.

4. El D2 (Tindle y otros) se refiere al uso de un antígeno de núcleo de Hepatitis B como un vehículo de entrega de los epítopos HPV de la proteína E7. El resumen del D1 indica que "puesto que las partículas HBcAg pueden administrarse en un adyuvante aceptable para aplicación humana... estos resultados tienen implicaciones para diseño de vacuna en cáncer anogenital asociado con HPV 16".

5. En el último parágrafo en la columna a mano izquierda, de la página 556, se amplía la referencia a adyuvantes. El documento dice: "en recientes documentos hemos obtenidos títulos altos del anticuerpo E7 en ratones inmunizados con partículas quiméricas HBcAg – E7 en algamulin.... y adyuvantes de alhidrogel"

6. Algamulin contiene inulina y alumbre gamma. Alhidrogel se refiere al alumbre adyuvante. Se sabe que el alumbre provoca una fuerte respuesta TH2, pero no una respuesta TH1. Por lo tanto, partiendo del D2, la persona versada en la técnica estaría refiriéndose a adyuvantes TH2 y no a adyuvantes TH1. Es decir, la persona versada en la técnica no combinaría las enseñanzas del D1 con las del D2. Dicha combinación se haría únicamente con el conocimiento de la presente invención y representa análisis *ex post facto*, lo cual es permisible.

7. Sírvanse observar que la persona versada en esta materia no tiene ninguna motivación para combinar ciertos antígenos definidos con un adyuvante TH1 a la luz de las enseñanzas de Tindle.

8. Teniendo en cuenta lo dispuesto por el Consejo de Estado en sentencia proferida dentro del Expediente No. 7684 el 22 de Octubre de 2004 y acogida por esa Superintendencia mediante memorando interno de fecha 22 de Diciembre de 2004 dirigido a los funcionarios de la Delegatura de Propiedad Industrial, no se hace necesario acompañar comprobante de pago por concepto de modificación de la solicitud por cuanto el nuevo pliego reivindicatorio se presenta a instancia de la Superintendencia en cumplimiento de lo ordenado por el artículo 45 de la Decisión 486.

Renuncio al término faltante de traslado, y les solicito otorgar el privilegio de patente de invención aquí tramitado.

De Ustedes Atentamente,

GERMAN CASTILLO GRAU
T.P. No. 2.978 del C.S.J.

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VIROLOGY 200, 547–557 (1994)

Chimeric Hepatitis B Core Antigen Particles Containing B- and Th-Epitopes of Human Papillomavirus Type 16 E7 Protein Induce Specific Antibody and T-Helper Responses in Immunised Mice

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The use of hepatitis B core antigen (HBcAg) as an immunogenic delivery vehicle for foreign epitopes has been reported. Here we report the insertion of DNA sequences encoding immunodominant linear B-epitopes and a "universal" T-helper epitope of human papillomavirus (HPV) type 16 E7 transforming protein into the full-length HBcAg gene cloned into an inducible bacterial expression plasmid (pPN1.0). The resulting chimeric proteins assembled into particles which were highly immunogenic. Mice immunised with particles containing one or two of the three E7 B-epitopes under consideration produced strong epitope-specific antibody responses with both IgG and IgG2a components which recognised eukaryotic E7. T-proliferative responses were elicited to the E7 T-epitope as well as HBcAg T-epitope(s). Lymph node cells from immunised mice produced IL-2 and IL-4 when specifically recalled *in vitro*, indicating stimulation of both Th_1 and Th_2 helper cell compartments. Since HBcAg particles can be administered in adjuvant acceptable for human application and can elicit mucosal responses after nasal or oral immunisation, these results have implications for vaccine design in HPV16-associated anogenital cancer. © 1994 Academic Press, Inc.

INTRODUCTION

Human papillomavirus (HPV) genotypes 6, 11, 16, 18, 33, 35, and 42 constitute a subgroup of HPVs infective for anogenital epithelium (Pfister, 1984; DeVilliers *et al.*, 1989). HPV16 DNA is found in about 50% of cervical squamous cell carcinomas (SCCs), and this association has raised the possibility of a causal role for HPV16 in cervical carcinogenesis (Lebeit *et al.*, 1992). Progression to malignancy is associated with production of E7 and E6 viral early open reading frame (e-orf) cell-transforming proteins (Hawley-Nelson *et al.*, 1989; Matlashewski *et al.*, 1987; Yasumoto *et al.*, 1986). Circumstantial evidence implicates host immune mechanisms in the control of HPV-associated tumours (for review, see Frazer and Tindle, 1992), and there is an increased risk of SCC of the cervix, but not of "control" organs (e.g., breast, rectum) in immunosuppressed allograft recipients (Rudlinger *et al.*, 1986). Antibodies reacting with HPV 16 E7 protein are detected up to 14× more frequently in the serum of cervical carcinoma patients than those with non-malignant infection or condylomata (Jochmus-Kudielka *et al.*, 1989). The E7 protein functioned as a tumour rejection antigen in mice immunised with E7-transfected fibroblasts and subsequently challenged with E7-transfected melanoma cells (Chen *et al.*, 1991). E7 is thus a candidate antigen for inclusion in a therapeutic vaccine for cervical cancer. We have previously defined three major murine B-epitopes (Tindle *et al.*, 1990) and a T-helper (Th) epitope (Tindle *et al.*, 1991) in the E7 protein of HPV16. In addition we have linked B-epitopes and the Th-epitope in synthetic peptides which elicit moderate antibody responses when used to immunise mice in complete Freunds adjuvant (CFA) (Tindle *et al.*, 1991).

We have been concerned to maximise the antibody responses to these E7 subunit vaccine peptides. In the present study we have used the bacterial plasmid pPN1.0 (=pPV *Nhe*I (Brown *et al.*, 1991) which contains the entire hepatitis B virus core antigen (HBcAg) gene under the influence of a *Tac* promoter, to make three chimeric HBcAg-E7 proteins which expressed one or more B-epitopes of E7. In addition, two of the chimeric proteins contained the E7 Th-epitope.

The use of HBcAg as a delivery vehicle for foreign epitopes has several advantages (Brown *et al.*, 1991). The protein subunit is a 21-kDa polypeptide that spontaneously assembles into 27-nm particles which are powerful immunogens (Hoofnagle *et al.*, 1973) because of their polymeric nature and the presence of a number of Th- epitopes within the HBcAg sequence

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(Millich *et al.*, 1987). Peptide -HBcAg fusion particles are more immunogenic than the corresponding peptide-carrier conjugates (Francis *et al.*, 1990).

Two of the three chimeric proteins reported here were highly immunogenic at low doses when used to *immunise mice*, eliciting antibody responses specifically recognising E7 peptides containing the corresponding B-epitopes, and the whole E7 protein. *In vitro* proliferation and interleukin-2 (IL-2) and interleukin-4 (IL-4) production assays indicated the induction of both Th-1 and Th-2 type responses, respectively, a supposition supported by the observation of both IgG2a and IgG1 components of the specific antibody responses.

Since HBcAg particles can be administered in adjuvant acceptable for human application and can elicit responses after nasal or oral immunisation (Francis *et al.*, 1990), the present findings have implications for HPV16 vaccine design.

MATERIALS AND METHODS

Genetic manipulations

Recombinant DNA manipulations were carried out using plasmid pPN1.0 which contains a *Nhe*I restriction site mutated into the e1 region of the HBcAg sequence which is under the transcriptional control of a *Tac* promoter. To construct chimeric fusion particles containing specific epitopes of the HPV16 E7 protein, synthetic oligonucleotides flanked by *Nhe*I cohesive ends were prepared using an Applied Biosystems 381A DNA synthesizer (sequences available upon request). Following annealing the oligonucleotides were ligated into *Nhe*I-digested pPN1.0 which had been purified from agarose using standard methods. *Escherichia coli* XL-1 blue were transformed with ligated DNA.

Colony screening assay

Expression of chimeric proteins in bacterial colonies on ampicillin-agar plates was induced by addition of isopropyl-B-D-thiogalactoside (IPTG; 100 μg/ml final concentration). Colonies expressing HPV16 E7 B-epitopes were detected on replicate nitrocellulose blots using a panel of HPV 16 E7 epitope-specific monoclonal antibodies (MAbs; below). Specific binding was detected using ^{125}I-anti-mouse Ig (Amersham, UK) and autoradiography on X-OMAT film (Kodak).

Purification of fusion particles

Cultures (500 ml) of bacteria harbouring recombinant plasmids were grown in ampicillin/LB broth and induced with IPTG as described (Clarke *et al.*, 1990) Pelletted bacteria were lysed and particles were extracted (Stahl *et al.*, 1982) and purified on 15–45% sucrose density gradients as described (Clarke *et al.*, 1990). Gradients were fractionated into 2-ml aliquots which were analysed spectrophotometrically and by immunoblot using HPV16 E7 epitope-specific MAbs. Peak fractions were pooled and dialysed against phosphate-buffered saline (PBS), pH 7.4, to remove sucrose and the particles were concentrated by centrifugation (110,000 *g*, 4°, SW28 rotor 16 *hr*). The pelletted particles were resuspended in PBS; examined by electron microscopy, and formulated for vaccine preparation.

Analysis of chimeric products

Bacterial lysates and purified particles were analysed on 12.0% reducing SDS–polyacrylamide (SDS–PAGE) gels. Proteins were visualised by Coomassie blue staining and by Western blotting using anti-HBcAg-specific antiserum and HPV 16 E7 epitope-specific MAbs. Blots were developed by incubation with anti-species Ig conjugated to horseradish peroxidase (Silenus Pty, Australia) and visualised using diaminobenzidine tetrahydrochloride (DAB) (Sigma) with hydrogen peroxide.

Epitopes and antibodies

Three immunodominant linear B-epitopes of the HPV 16 E7, ^{10}EYMLD^{14} (*single letter amino acid code*), ^{38}IDGP^{41}, and ^{44}QAEPD^{48} were defined by mouse MAbs 8F, 4F, and 6D, respectively (Tindle *et al.*, 1990). ^{49}DRAHYNI^{54} is a universal Th-epitope of HPV16 E7 capable of providing "help" for antibody production to any of the B-epitopes, singly or in combination (Tindle *et al.*, 1991).

Peptides

HPV 16 E7 peptides were synthesised using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 431A peptide synthesiser. The HPLC-profile, amino acid composition, toxicity, and mitogenicity of all peptides were checked.

Baculovirus E7

A full-length E7 gene cloned into baculovirus intermediate vector pcYM1 was cotransfected into *Spodoptera frugiperda* cells with wild-type virus to yield polyhedrin gene recombinants which produced eukaryotic E7 when infected into "second round" *Spodoptera* cells (Park *et al.*, 1994). Lysates from E7 containing cells were separated on SDS–PAGE, transferred to nitrocellulose, and immunoblotted with sera from immunised mice, or E7-specific MAbs. Specific bands were visualised with ^{125}I-anti-mouse Ig and autoradiography on X-OMAT film.

Mice

Inbred mouse strains were obtained from the University of Queensland animal breeding facility or from Ani-

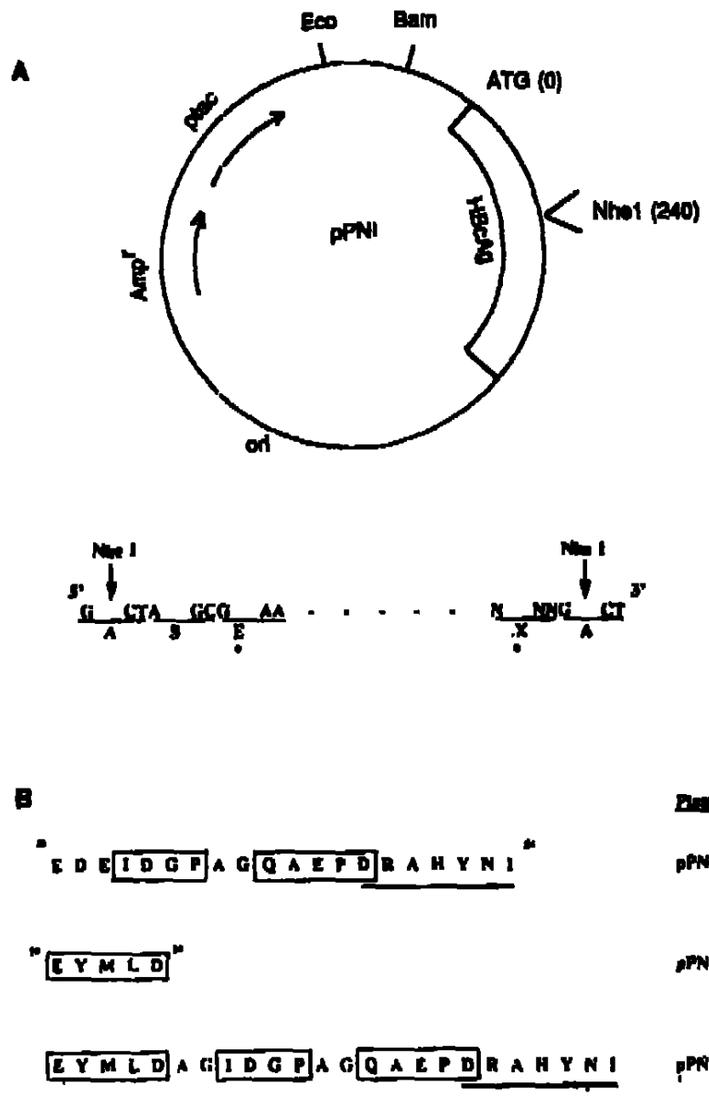

FIG. 1. (A) Schematic portrayal of plasmid pPN1.0. The nucleotide sequences surrounding the *Nhe*I site and the translational reading frame are shown. *The first and last residues encoded by HPV16 E7-derived sequences. (B) Amino acid sequences encoded by HPV16 E7 inserts and flanking regions of 3 recombinant plasmids pPN1.1, pPN1.15 and pPN1.27. HPV16.E7 B-epitopes are boxed. HPV16 E7 Th-epitope is underlined.

mal Resources Centre (Perth, Western Australia). Mice were used at 8–24 weeks of age. Genetic purity was checked routinely by isoenzyme analysis.

Immunisation for antibody

Mice were immunised intramuscularly (im) with purified wild-type or recombinant HBcAg particles in incomplete Freunds adjuvant (IFA) on Days 0 and 56 (study 1) or 49 (study 2). Mice were bled from the retro-orbital plexus at intervals, and serum was prepared and tested in ELISA against peptides containing appropriate E7 B-epitope sequences.

Peptide ELISA assay

Peptides were bound to microtitre plates by incubation at 10 μg/ml in PBS, pH 7.4. Remaining binding

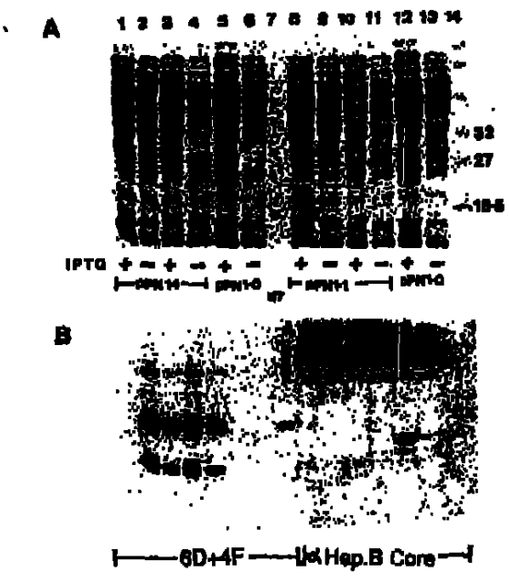

FIG. 2. SDS–PAGE (12.0%) of lysates of *E. coli* XL-1 blue cells containing HBcAg plasmids. (A) Coomassie blue-stained gel of IPTG induced (lanes 1, 3, 8, 10) and uninduced (lanes 2, 4, 9, 11) bacteria containing pPN1.1; IPTG induced (lanes 5, 12) and uninduced (lanes 6, 13) bacteria containing pPN1.0, E7/M52 replicase fusion protein (lane 7) and MW markers (lane 15). B. Western blot analysis of a duplicate gel using MAb mix 6D + 4F (lanes 1–7), or guinea pig anti-HBcAg antiserum (lanes 8–13).

sites were blocked with PBS/0.25% gelatin/0.1% Tween 20. Binding and antigenic integrity of bound peptides were confirmed using Mabs 6F, 4F, and 6D. ELISA assays on these coated plates were conducted as described previously (Tindle *et al.*, 1991). Briefly the plates were incubated with serial dilutions (in PBS/0.125% gelatin/0.05% Tween 20) of sera from immunised mice, and anti-mouse Ig conjugated to horseradish peroxidase, with appropriate washing steps. Reaction was visualised by addition of substrate (2,2'-azinobis (3-ethylbenzthiazoline) sulphonic acid) and chromogenicity monitored at 414 n*M* on a Titertek automatic plate reader. In isotype specific assays, heavy chain-specific anti-mouse IgG2a and anti-mouse IgG1 replaced anti-mouse Ig.

Lymph node cell (LNC) proliferation assays

Proliferation assays were carried out as described (Tindle *et al.*, 1991). Briefly, C57BL/6 mice were immunised subcutaneously (sc.) at the base of the tail with 6 μg purified wild-type or recombinant HBcAg particles emulsified in CFA (Difco R37). Eight to 10 days later a suspension of draining LNCs was prepared and a 4-day proliferation assay was done in peptide-coated microtitre plates. Proliferation was quantified by [^{3}H]-thymidine incorporation over 6 hours.

Assay for cytokine production

Supernatants from LNC proliferation assays were harvested at 4 days and tested for their ability to induce proliferation of the interleukin-3 (IL-3) dependent cell line FDC-P1 (Dotsika and Sanderson, 1987), the IL-2-dependent cell line CTLL (Gillis *et al.*, 1978) and the IL-4-dependent cell line CT-4S (Hu-Li *et al.*, 1989), as measured by [^{3}H]thymidine incorporation over 6 hr.

RESULTS

HBcAg-E7 particles

Three sequences were selected to encode the immunogenic regions of the HPV16 E7 protein. (Fig. 1B). The first sequence encoded two of the three B-epitopes (IDGP and QAEPD), and the minimal Th-epitope (DRAHYNI). The second sequence encoded the third B-epitope (EYMLD) only. The third sequence encoded all 3 B-epitopes and the Th-epitope.

The plasmid vector pPN1.0 contains the entire HBcAg gene into which has been mutated a *Nhe*I restriction site (Brown *et al.*, 1991), in a region which encodes the immunodominant α1 surface loop region of the particle (Fig. 1A). Synthetic oligonucleotides coding for the amino acid sequences shown in figure 1B were inserted into pPN1.0 at the *Nhe*I site to produce plasmids pPN1.1, pPN1.16 and pPN1.27. Clones identified as expressing insert in a colony screening assay using E7 epitope-specific MAbs, were grown in the presence of IPTG to produce recombinant antigen and samples from the cell lysates of these bacteria were analysed by SDS–PAGE and Western blotting. Figure 2A shows a Coomassie blue-stained gel from two individual colonies containing recombinant plasmid pPN1.1. A novel protein of the expected *M*, (28 kDa) was apparent in bacteria containing pPN1.1 (lanes 1–4; arrowed in lane 3) but not in bacteria containing the parent plasmid pPN1.0 which produce non-recombinant HBcAg protein of *M*, 24 kDa (lanes 5, 6; arrowed in lane 5). (Note that plasmid encoded proteins are also expressed at a lower level in bacteria not grown in IPTG. This presumably results from "leaky" expression from the *Tac* promoter in the XL-1 blue strain of *E. coli*.) The induced protein in recombinant lysates but not parent lysates reacted with an antibody probe consisting of a mixture of 6D and 4F MAbs, confirming the presence of E7-derived amino acid sequences (Fig. 2B, lanes 1–4). The major immunode-

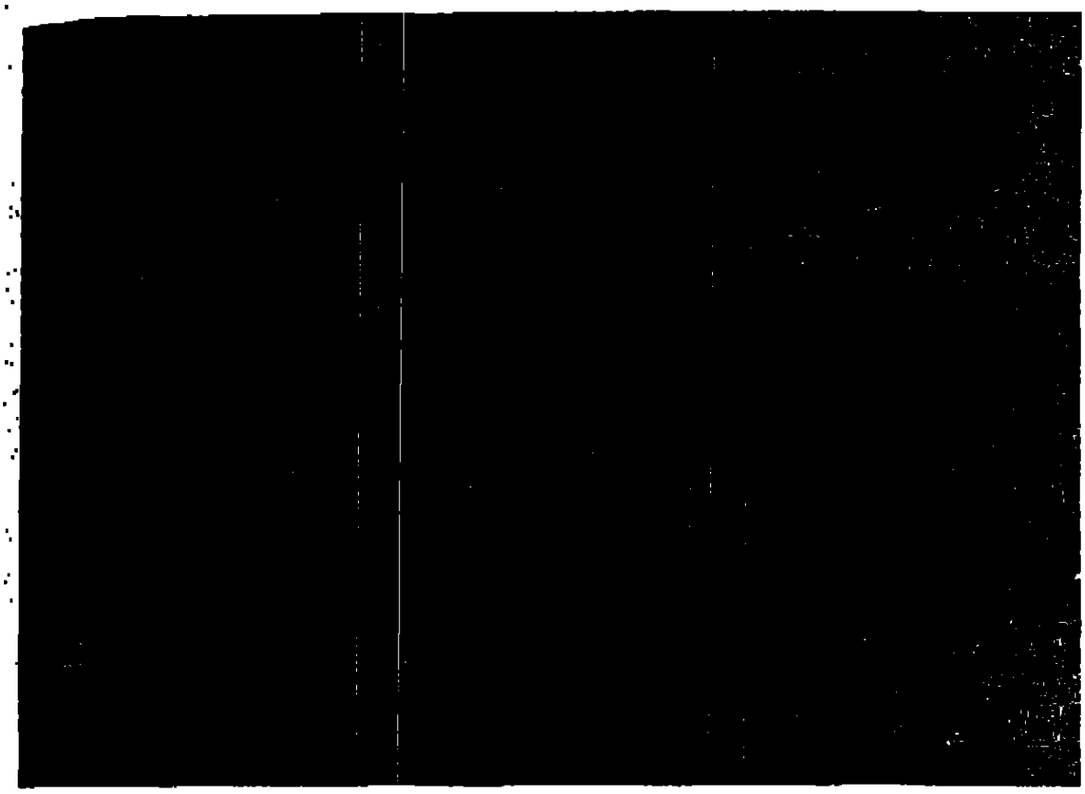

FIG. 3. Electron micrograph (×48,000) of core particles produced from plasmid pPN1.1.

tected band was seen at ca.28 kDa. A second band was also detected at ca.20 kDa which may represent a carboxy-terminal cleavage product of the recombinant HBcAg (Clarke *et al.*, 1990) which nonetheless contained intact E7 B-epitopes. While nonrecombinant HBcAg in parent lysates was recognised by anti HBcAg antiserum (Fig. 2B, lane 12), HBcAg recombinant for E7 sequence was not (Fig. 2B, lanes 8–11), indicating that insertion destroys epitope(s) within HBcAg.

Cultures (500 ml) of XL-1 blue cells containing pPN1.1, pPN1.15, or pPN1.27 plasmids were pelletted and the assembled core-particles purified from bacterial lysates as described (Clarke *et al.*, 1990). Concentrated peak fractions from the sucrose gradients contained particulate structures visualised by electron microscopy. Those of pPN1.1 are shown in Fig. 3. Core particles from pPN1.0, pPN1.15, and pPN1.27 were morphologically identical to those from pPN1.1. Purified particles were analysed by SDS–PAGE and Western blotting (Fig. 4). Particles produced by pPN1.1 plasmid reacted with MAbs 4F and 6D which recognise E7 B-epitopes IDGP and QAEPD, respectively, and particles produced by pPN1.15 reacted with MAb 8F which recognises E7 B-epitope EYMLD. Particles produced by pPN1.27 reacted with 4F, 6D, and 8F, indicating that all three B-epitopes were present. Irrelevant MAbs were negative on all particles. Particles produced by parent plasmid pPN1.0 reacted with none of the MAbs.

Antibody induction

Sera collected at Day 71 from mice immunised with 3.6 μg of recombinant core particles in IFA on Days 0 and 49 were reacted in ELISA against HPV16 E7 peptides 101 (MHGDTPTLHEYMLDLQPE), 104 (YEQLND-SSEEEDEIDGPAG), and 106 (GQAEPDRAHYNIVTF-CCKCD), which contain the B-epitopes EYMLD, IDGP, and QAEPD respectively (underlined). Sera from 4/4 mice immunised with pPN1.1 particles reacted with peptide 104 and 3/4 reacted with peptide 106. Sera from none of these mice reacted with peptide 101. (Figs. 5A–5C). Sera from 4/4 mice immunised with pPN1.15 particles reacted with peptide 101, but not with peptides 104 or 106. None of the sera from mice immunised with pPN1.27 reacted with any of the three

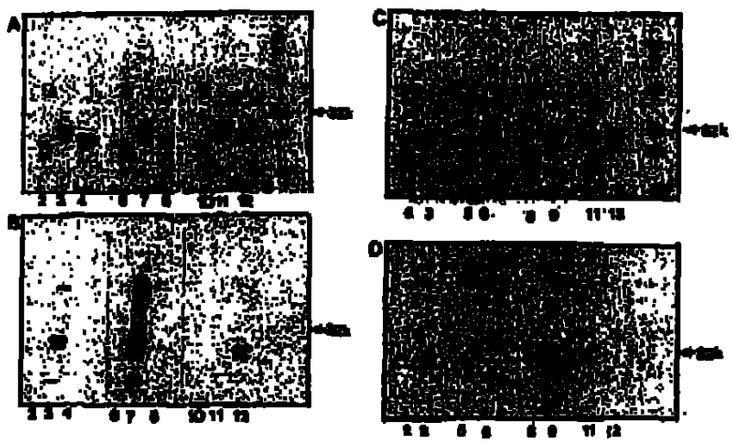

FIG. 4. Coomassie blue-stained gels of purified HBcAg particles A.pPN1.0 (lanes 2, 6, 10), pPN 1.1 (lanes 3, 7, 11), pPN1.15 (lanes 4, 8, 12), and MW markers; C.pPN1.0 (lanes 2, 5, 8, 11), pPN1.27 (lanes 3, 6, 9, 12). Western Blots of duplicate gels stained with B.MAbs 4F (lanes 2, 3, 4), 6D (lanes 6, 7, 8) and 8F (lanes 10, 11, 12): D. Mabs 8F (lanes 2, 3), 4F (lanes 5, 6) 6D (lanes 8, 9) and irrelevant antibody (lanes 11, 12).

peptides, although all reacted with HBcAg, indicating that the mice had been effectively immunised (data not shown). Sera from mice immunised with pPN1.1 in saline failed to react with any peptide. Sera from mice immunised with 6 ug of peptide 105 (IDGPAGQAEPDRAHYNI) containing B-epitopes IDGP and QAEPD and Th-epitope DRAHYNI, in CFA, also failed to react with any peptide.

To determine the time course and maintenance of immune response induction, serum from mice immunised on Days 0 and 56 with 3.6 μg pPN1.1 in IFA were sampled at intervals for ELISA reactivity to HBcAg and to peptide 105 (Fig. 6D). Although high-titre antibody to HBcAg was apparent at Day 28 after first immunisation and persisted at least until Day 63 when evaluation ceased, high titre antibody to peptide 105 was not detected until Day 83, 7 days after the second immunisation. High antibody titres to peptide 105 were still apparent at Day 133.

The contribution of IgG1 and IgG2a subclasses to the E7-epitope-specific antibody response of pPN1.1 immunised mice was investigated by using HRPO anti-gamma 1 and anti-gamma 2a heavy-chain-specific "second" antibodies as detectors in ELISA reactions of sera with peptide 105 (Table 1). Of the seven sera used in this experiment, six contained IgG1 and three contained IgG2a antibodies reactive with peptide 105, respectively (positive response defined as $OD_{414} > 0.1$ at 1:200 dilution). The ELISA absorbance readings of the 3 IgG2a positive sera were considerably lower than those of the IgG1 positive sera. Controls indicated that the efficacy as ELISA developing reagents of the HRPO anti-gamma 2a and HRPO anti-gamma 1 for equivalent amounts of mouse IgG2a and mouse IgG1 were comparable (not shown).

Antibodies recognise eukaryotic E7

To ascertain whether immunisation with HBcAg particles recombinant for E7 B-epitopes produced antisera which recognised whole eukaryotic E7 protein, sera were used to immunoblot E7 from lysates of *Spodoptera frugiperda* cells infected with baculovirus recombinant for HPV16 E7 protein. Sera from pPN1.1 immunised mice but not pre-immune sera, detected a band at the same position as that detected by the anti-E7 MAb 6D (Fig. 6).

T-proliferation and cytokine production

To demonstrate Th- epitopes within the HBcAg-E7 particles, draining LNC from mice immunised with pPN1.1, pPN1.15, or peptide 106 were assayed for proliferation and cytokine secretion upon challenge with pPN1.0 and peptide 106 (which contains the E7 Th epitope DRAHYNI (Tindle *et al.*, 1991)). LNC from pPN1.1 and 106 immunised mice specifically proliferated when challenged with peptide 106, and LNC from pPN1.1 and pPN1.15 immunised mice specifically proliferated when challenged with pPN1.0 (Figs. 7A and 7B). These data indicate that the C57BL/6 (H-2b) mouse T-cell repertoire (a) was primed by the murine Th-epitope DRAHYNI present in pPN1.1 particles since specifically primed cells could be recalled by E7 peptide containing DRAHYNI and (b) was primed by T epitope(s) in HBcAg, since specifically primed cells could be recalled with non-recombinant pPN1.0 parent particles. In a further experiment, (Figs. 7C, 7D, and 7E) supernatant fluids of proliferating LNC from pPN1.1 immunised mice recalled *in vitro* with pPN1.0 were added to cytokine-starved cell lines CTLL, FDC-P1, and CT-4S which are critically dependent upon IL-2.

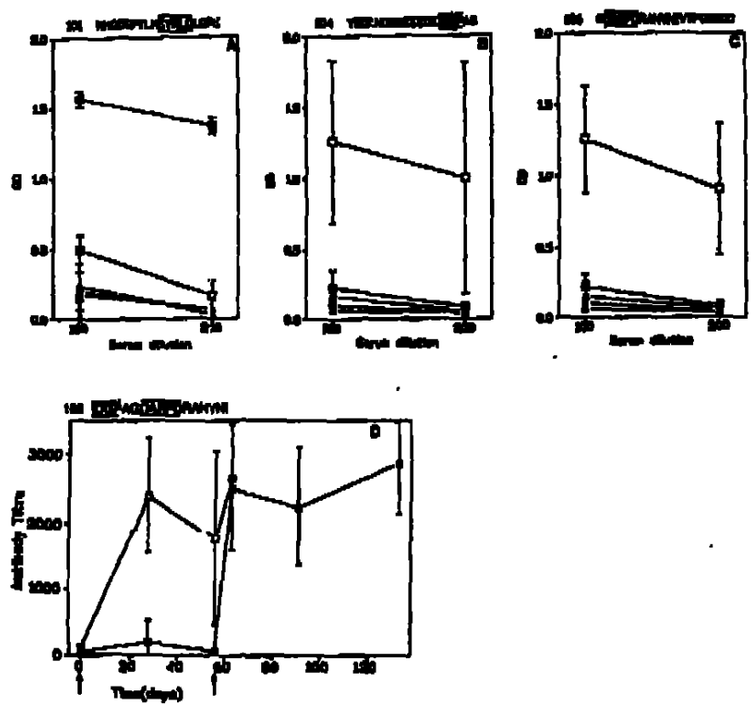

FIG. 6. (A–C) ELISA reactivity of sera from mice (C57BL/6; 4/group) immunised with pPN1.1 in IFA (open squares), pPN1.1 in saline (open diamonds), pPN1.16 in IFA (closed squares), pPN1.27 in IFA (open triangles), and pPN1.0 in IFA (closed triangles) against HPV 16 E7 peptides containing B-epitopes EYMLD (A), IDGP (B) or QAEPD (C). Data points are arithmetic means of reactive mice ± SD. (D) ELISA titers (arithmetic mean ± SD) of sera from mice (Balb/C) immunised with pPN1.1 in IFA on Days 0 and 56 (arrows) and collected on Days 0, 28, 56, 63, 91, and 133 against HBcAg (open squares) or peptide 105 (closed squares).

IL-3, and IL-4, respectively, for proliferation. These supernatants induced the proliferation of CTLL, FDC-P1, and CT-4S cells, whereas control supernatants did not. These data indicate that T-epitopes in HBcAg stimulate polyclonal T cell populations to secrete IL-2, IL-3, and IL-4. These cytokines are implicated in Th cell-driven immune responses.

TABLE 1

Ig. Subclass Response of Mice Immunised with Recombinant HBcAg Particles

Immunogen	Mean serum ELISA reactivity ± SD[a] (number of reactive mice)	
	IgG_1	IgG_{2a}
pPN1.1	1.45 ± 0.32 (6/7)	0.20 ± 0.08 (3/7)
pPN1.0	0.02 ± 0.003 (0/5)	0.01 ± 0.007 (0/5)

Note. Sera taken from mice 83 days after immunisation on Days 0 and 56 were reacted in ELISA assay with peptide 105. Ig subclass-specific binding was determined using anti-gamma 1- or anti-gamma 2a-specific HRPO-conjugated antisera.

[a] OD414, 1/200 serum dilution.

DISCUSSION

The susceptibility of naturally or iatrogenically T-immunosuppressed individuals to progression of HPV 16 associated lesions indicates immune system involvement in control of disease and suggests that a vaccine approach to therapy may be feasible (discussed in Tindle and Frazer, 1993). E7 is the most abundant viral protein produced in premalignant and malignant HPV 16-associated lesions (Smotkin and Wettstein, 1987). Its candidature as a target for vaccine mediated immunomodulation is reinforced by the demonstration of E7 as a putative tumour-associated antigen (Chen *et al.*, 1991).

Our previous work has shown that synthetic peptides composed of linked B-epitopes and a Th-epitope of HPV 16 E7 protein elicit moderate antibody responses which recognise the intact E7 molecule, when administered in CFA to mice (Tindle *et al.*, 1991). In the present study, we investigated the possibility of enhancing immunogenicity by presenting the epitopes as fusion proteins with HBcAg. Recombinant HBcAg fusion proteins have been expressed in a range of sys-

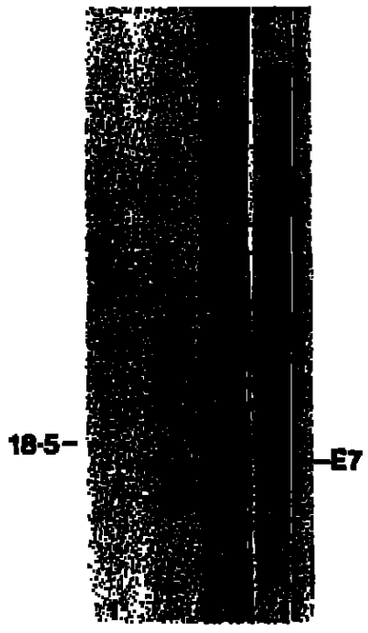

FIG. 6. Western Blot of *S. frugiperda* cells infected with baculovirus recombinant for the whole E7 protein of HPV16. Lysates from infected cells were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with irrelevant MAb (lane 1), 8D MAb (lane 2), pooled pre-immune mouse serum (lane 3) pooled sera from mice immunised with pPN1.1 particles (lane 4).

tems other than bacteria, including yeast (Kniskern *et al.*, 1988), mammalian cells (Roosinck *et al.*, 1986), vaccinia virus (Clarke *et al.*, 1987), and baculovirus (Lanford *et al.*, 1989). In bacteria, heterologous sequences have been inserted N-terminal to the HBcAg gene (Clarke *et al.*, 1990) or at a *Nhe* site genetically engineered into the e-coding region within the gene (Brown *et al.*, 1991). Clarke *et al.*, (1990) reported expression and immunogenicity of three viral B-epitopes (from poliovirus type 1, feline leukaemia virus, and human rhinovirus, respectively) encoded by sequences N-terminally flanking the HBcAg gene in a *Tac*-driven bacterial expression plasmid. However, immunological responses to the human rhinovirus epitope were 10-fold greater when the coding sequences were inserted into the *Nhe*I site within the HBcAg a-region (Brown *et al.*, 1991). Structural predictions indicate that the e-region product should be present as a surface located loop within the HBcAg particles (Argos and Fuller, 1988) which is a likely reason for the immunodominance of this region (Brown *et al.*, 1991).

We made three recombinant constructs each composed of DNA encoding one or more HPV 16 E7 epitopes inserted into the e-region of HBcAg under the influence of the *Tac* promoter in bacterial plasmid pPN1.0. The chimeric HBcAg-E7 proteins were expressed to high levels in bacteria, and the individual E7 B-epitopes were easily immunodetectable. The chimeric proteins assembled into particles morphologically indistinguishable from those described for native core antigen (Stahl *et al.*, 1982) and which could be substantially purified from bacterial proteins on sucrose density gradients.

Plasmid pPN1.1 encoded E7 residues 35 to 54 containing two B-epitopes IDGP and QAEPD and the Th-epitope DRAHYNI in the natural linear configuration in which they occur in the whole E7 molecule. pPN1.1 particles induced antibody to both the B-epitopes when administered to mice. Mean responses of groups of mice measured against IDGP-containing or QAEPD-containing peptides were similar, though in some individual mice, one or other of the epitopes was preferentially recognised (data not shown). The E7 DRAHYNI Th epitope was also recognised by mice as evidenced by proliferation of LNC from pPN1.1 immunised mice when challenged *in vitro* with peptide containing the DRAHYNI sequence. Ideally, subunit HPV vaccines would be composed of T- and B-cell epitopes derived from the same HPV protein so that latent and/or subsequent natural infections would elicit a response from both populations, which is critical if T-B co-operation is required for protection. A proliferative response by LNC from pPN1.1 particle immunised mice was elicited by parent HBcAg (pPN1.0) recall, supporting the observation of active Th-epitopes within HBcAg (Milich *et al.*, 1987). The mitogenic response generated *in vitro* was shown to be due to stimulation of T-cells, as the culture supernatants from these cells were able to stimulate the IL-3-dependent cell line FDC-P1. The present experiments do not determine whether the DRAHYNI T-epitope or T-epitopes within HBcAg or both provided help for the observed antibody responses in pPN1.1 immunised mice. That mice immunised with pPN1.15 particles which do not contain an E7-derived T-epitope produced antibody indicates that T-help in this case was derived solely from HBcAg. Plasmid pPN1.15 encoded E7 residues 10 to 14 comprising B-epitope EYMLD and pPN1.15 particle immunised mice exhibited strong antibody responses to peptide containing EYMLD. Previously encountered limitations in eliciting antibody responses to small inserts (<9aa) in HBcAg, attributable to inefficient epitope exposure at the surface of the particle (Clarke, *et al.*, 1990) have apparently not applied to pPN1.15.

pPN1.27 was constructed in a attempt to deliver to the immune system all 3 B-epitopes and the E7 Th-epitope in a single recombinant particle. pPN1.27 encoded particles containing the EYMLD epitope connected N terminally through an AG linker to the alignment IDGPAGQAEPDRAHYNI, used in pPN1.1. No

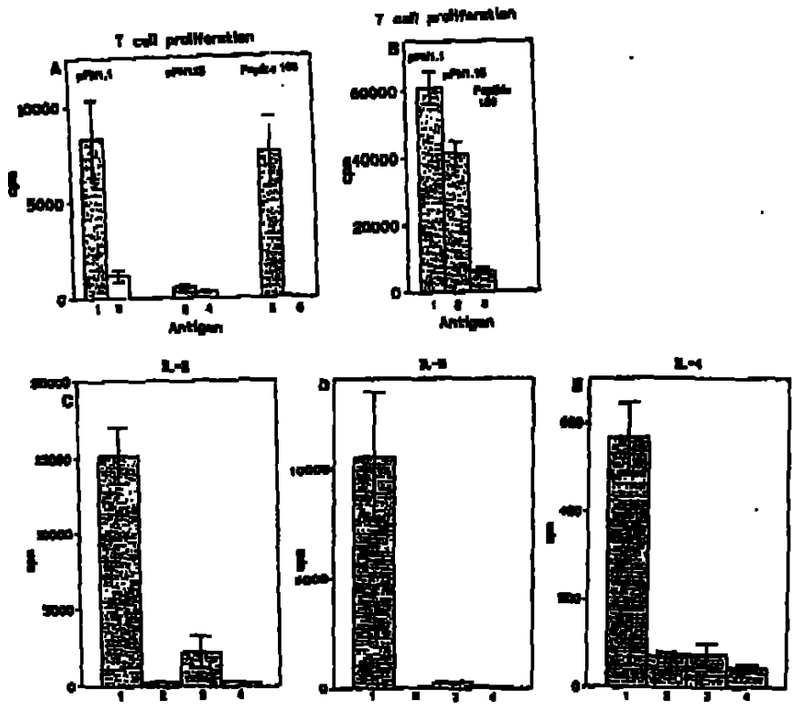

FIG. 7. The *in vitro* proliferative response (A, B) and lymphokine production (C, D, E) of LNC. (A) Pooled LNC from C57 BL/6 mice (4 per group) immunized with 6 μg pPN1.1 particles (bars 1, 2), 6 μg pPN1.15 particles (bars 3, 4), or 6 μg peptide 105 in CFA (bars 5, 6) were challenged *in vitro* with peptide 105 at 10 μg/ml (bars 1, 3, 5) or with tissue culture medium (bars 2, 4, 6). (B) Pooled LNC from C57BL/6 mice (4 per group) immunized with 6 μg of pPN1.1 (bar 1), 6 μg of pPN1.15 (bar 2), or 6 μg peptide 105 in CFA (bar 3) were challenged *in vitro* with pPN1.0 particles (5 μg/ml) (bars 1, 2, 3). Counts were means of [^{3}H]thymidine pulsed triplicate wells ± standard deviation. (C, D, E): Culture supernatants of LNC from mice immunized with 3.6 μg of pPN1.1 particles (bars 1, 2), or tissue culture medium (bars 3, 4), and challenged *in vitro* with 6 μg/ml of pPN1.1 particles (bars 1, 3), or tissue culture medium (bars 2, 4) were harvested at 4 days and added at 1:2 dilution to cytokine-starved reporter cell lines (A) CTLL (IL-2 dependent), (B) FDC-P1 (IL-3 dependent), and (C) CT 4S (IL-4 dependent). Wells were pulsed with 1 μCi [^{3}H]thymidine at 24 hr (CTLL and FDC-P1) or 72 hr (CT 4S) later. Wells were harvested at 6 hr. Results are arithmetic means of triplicate wells ± standard deviation. Background counts (tissue culture medium added instead of supernatant) were 498.5 ± 92 (for CTLL), 66.8 ± 22.7 (for FDC.P1), and 86.3 ± 10.2 (for CT-4S). Maximal stimulations effected by adding cytokine were 30,550 ± 1767 (IL-2 added to CTLL), 44,866 ± 1625 (IL-3 added to FDC P1), and 29,647 ± 667 (IL-4 added to CT-4S).

antibody was produced to peptides containing any of the three B-epitopes when mice were immunized with pPN1.27 particles. It is not immediately apparent why this was so, since Western blotting indicated that all three B-epitopes were intact and sucrose gradient banding and electron microscopy showed that the pPN1.27 protein assembled into particles similar to those expressed by the parent plasmid pPN1.0 and recombinant plasmids pPN1.1 and pPN1.15. Furthermore, the sera from pPN1.27 particle immunized mice contained antibody to HBcAg indicating that mice were adequately immunized and that particle Th epitopes were functional. Possibly, addition of EYMLDAG changes local e-loop configuration which precludes insert recognition by the host immune response.

Although comparative quantitative and time-course experiments were not part of this study, the immunogenicity of E7 B-epitopes presented in HBcAg chimeric particles was considerably greater than E7 B-epitopes presented as peptide administered in CFA. Three point 6 μg of pPN1.1 particles, equivalent to 0.21 μg (by molar ratio) of E7 sequence elicited strong antibody responses after two injections in IFA, 56 days apart, and furthermore responses lasted at least 77 days after the second injection. Six micrograms of free peptide 105 (i.e., the same sequence as that inserted in pPN1.1 particles) in CFA, identically administered with the same regimen, failed to elicit any anti-peptide response as detected by ELISA. Previous experiments (Tindle *et al.*, 1991) have shown that ca. 20–100 μg of free E7 peptide containing B-epitope(s) and DRAHYNI Th-epitope given three times in CFA are required to elicit antibody responses similar in magnitude to those reported here with 0.21 μg of E7 sequence as recombi-

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nant HBcAg particles. Furthermore, the free peptide antibody response fell away ca. 25 days after the third injection.

Bulk LNC from pPN1.1 particle-immunised mice were induced to secrete cytokines IL-2 and IL-4 when recalled *in vitro* with HBcAg (pPN1.0 particles). This indicates activation of Th-1 and Th-2 "arms" of the T cell response, respectively. This is supported by the finding of both IgG2a (Th-1 driven) and IgG1 (Th-2 driven) components of the antibody response to E7 B-epitopes, although the response is skewed to IgG1. The nature of the response may reflect the mode of exposure of the host immune system to the E7 B-epitopes, i.e., exogenously by injection, as much as the properties of the HBcAg particles themselves. Immunisation with HBcAg-E7 chimeric particles produced antibody which recognised eukaryotic E7 (produced by a baculovirus recombinant). Clearly this is of import were the particles to be incorporated into a vaccine. The role of antibody in the control of E7 expressing tumour cells, whether in humans (Jochmus-Kudielka *et al.*, 1989) or in experimental mouse models (Chen *et al.*, 1991; Griep *et al.*, 1993), is equivocal. Investigation of putative mechaniams, e.g., antibody-dependent cell-mediated cytotoxicity (ADCMC), awaits the development of an appropriate E7-expressing *in vitro* tumour cell model.

Since cytotoxic T-lymphocyte (CTL) responses which are primarily Th_1 driven may be more efficacious than antibody in controlling E7-expressing tumour cells, it may be necessary to express E7 sequences from HBcAg constructs intracellularly in order to target the MHC class 1 antigen presentation pathway. HBcAg particles have been expressed in mammalian cells (Rosslnck *et al.*, 1986) and via vaccinia virus (Clarke *et al.*, 1987). Recombinant HBcAg constructs, driven off a low oxygen tension inducible promoter *nirB*, and infectious for gut colonising *Salmonella typhimurium* live oral vaccine strain (Chatfield *et al.*, 1992) where they become intracellular, may provide the alternative route. This latter approach has the added advantage of mucosal immunity induction.

In this paper, we show that B- and Th- epitopes of the E7 transforming protein of HPV16 can be rendered highly immunogenic (relative to immunisation with epitope-containing peptides) when incorporated into HBcAg particles and administered to mice at low doses in the absence of CFA. In recent experiments we have obtained high E7-antibody titres in mice immunised with HBcAg-E7 chimeric particles in Algammulin (Cooper *et al.*, 1991) and Alhydrogel adjuvants (Tindle and Herd, unpublished), the latter of which is licensed for use in men. We suggest that this approach may have implications for vaccine design for HPV16 associated cervical cancer.

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Immunisation of mice using *Salmonella typhimurium* expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen

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Live vaccines based on BRD509, an attenuated S. typhimurium *(aroA, aroD) strain, were constructed that directed the expression of hepatitis B core antigen particles (HBcAg) (BRD969) or HBcAg harbouring human papillomavirus type 16 E7 protein sequences (BRD974), under the control of the* in vivo *inducible* nirB *promoter. These strains were used to orally or intravenously immunise different inbred mouse strains and humoral, secretory and cellular anti-E7 and anti-HBcAg responses were monitored. Both BRD969 and BRD974 induced anti-HBcAg humoral IgG responses following oral or intravenous immunisation of BI0 mice, although responses were higher in BRD969 immunised animals. IgG subclass analysis revealed a predominantly IgG2a response in these animals. BRD974, but not BRD969, induced anti-E7 humoral IgG responses. Anti-HBcAg (BRD969 and BRD974) and anti-E7 (BRD974) IgA responses were detected in the intestines of orally immunised mice. Anti-Salmonella but not anti-HBcAg or anti-E7 T helper cell responses were detected in mice immunised with BRD509, BRD969 and BRD974. Thus Salmonella vaccine strains can be used to efficiently deliver HBcAg and E7 epitopes to the mucosal and systemic immune systems. Copyright © 1996 Elsevier Science Ltd.*

Keywords: Papilloma; *Salmonella*; hepatitis B; virus core antigen

The development of cervical cancer is closely associated in humans with infection by certain types of human papillomavirus (HPV) such as HPV type 16 (HPV-16)[1–3]. The molecular process of progression from virus infection to invasive cancer is still poorly understood but the onset of malignancy is usually associated with the insertion of viral DNA into the host genome and enhanced expression of the HPV-16 E6 and E7 proteins. In cultured cell systems, expression of E6 and E7 is sufficient for immortalisation[4,5]. E7 is an acidic, zinc-binding phosphoprotein[6] detected in the cytoplasm[7] and nucleus of HPV-16-transformed cells[8]. The E7 protein interacts with retinoblastoma tumour suppressor protein (pRB), and impairs its function as a cell growth regulator[9].

Since expression of E7 is closely associated with the neoplastic phenotype, this protein is considered to be a potential target for immunotherapy. E7-specific antibodies have been detected in the sera of patients suffering from cervical cancer[10,11]. An immunodominant region of the HPV-16 E7 protein has been mapped between amino acid residues 38–57 with two different B-cells[12,13], a universal helper T-cell[12,14,15] and a CTL epitope[16] clustered together (*Table 1*). Immunisation with synthetic peptides corresponding to this region induces humoral, helper and cytotoxic anti-E7 responses in mice[14,16] although repeat immunisation and the use of potentially toxic adjuvants are required.

Challenge studies in mice suggest that immunisation can induce selective destruction of HPV-induced tumours. For example, immunisation with a fibroblast cell line expressing the HPV-16 E7 protein can induce a tumour rejection response against a syngeneic HPV-16 E7 transfected melanoma cell line. This response is

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Table 1 The immunodominant region of HPV-16 E7

Consensus (30–62)	D	S	S	E	E	E	D	E	I	D	G	P	A	G	Q	A	E	P	D	R	A	H	Y	N	I	V	T	F	C	C	K	C	D[a]
	30					35					40					45				50						55					60		
B cell[b]									I	D	G	P			Q	A	E	P	D														
Th[c]																			D	R	A	H	Y	N	I								
CTL[d]																				R	A	H	Y	N	I	V	T	F					
16E7[e]						E	D	E	I	D	G	P	A	G	Q	A	E	P	D	R	A	H	Y	N	I								
P838[f]		D	S	S	E	E	E	D	E	I	D	G	P	A	G	Q	A	E	P	D	R													
8Q[g]															G	Q	A	E	P	D	R	A	H	Y	N	I	V	T	F	C	C	K	C	D

[a]Amino acid residues 30–62 of the HPV-16 E7 protein. Single letter amino acid codes are used. [b]The two B-cell epitopes are 38–41 and 44–48[19,42]. [c]The promiscuous helper T-cell epitope covers amino acid 48–64[10,34,41]. [d]The H-2[b]-restricted CTL epitope (49–57) was identified by Feltkamp *et al.*[14] [e]The amino acid sequence 38–54 of HPV-16 E7 protein was inserted into the e1 loop of HBcAg. [f]Synthetic peptide P838 covers amino acid residues 30–49 of the E7 protein. [g]Synthetic peptide 8Q covers amino acid 43–62 of the E7 protein

mediated by $CD8^+$ T lymphocytes[17]. Vaccination of mice with an E7-derived peptide containing a H-2^b restricted CTL epitope conferred protection in C57Bl/6J mice (H-2^b mice) against tumours induced by HPV-16 transformed cells[16].

Salmonella vaccine strains can establish self-limiting infections and induce potent immune responses that include mucosal and systemic antibodies and T-cell mediated helper and cytotoxic responses[18–20]. The nucleocapsid of the hepatitis B virus (HBV), also known as core antigen (HBcAg), has been used as a carrier for peptide sequences derived from HBV and other pathogens[21]. This particulate antigen is composed of approximately 180 subunits of a 21 kDa polypeptide. Insertion within HBcAg can enhance the immunogenicity of peptide sequence, especially when the insertions are in the immunodominant region of the HBcAg polypeptide known as the e1 loop[22,23]. In this report we describe the construction of *S. typhimurium* strains which express either HBcAg or chimeric $E7_{35-54}$/HBcAg particles under control of the *nirB* promoter. We demonstrate the stability and persistence of these strains *in vivo* as well as their immunogenicity in a mouse model.

MATERIALS AND METHODS

Oligonucleotides and DNA modification techniques

The synthetic oligonucleotides employed in this study were synthesised using an Applied Biosytems 392 DNA/RNA synthesiser. Restriction and modification enzymes were purchased from Boehringer Mannheim (Lewes, East Sussex, UK) and used following the manufacturer's recommendations. The DNA Taq polymerase used in Polymerase Chain Reactions (PCR) reactions was obtained from Perkin Elmer Cetus (Stoke, Staffordshire, UK) and used in DNA amplifications following the supplier's recommended conditions. Other DNA manipulation techniques were according to[24].

Bacterial strains and growth conditions

E. coli HB101[24] served as a host for DNA manipulation experiments. *S. typhimurium* LB 5010[25] was used as an intermediate strain to prepare plasmid DNA for introduction into *Salmonella* vaccine strains. *S. typhimurium* BRD509 has been described previously[26]. All the bacterial strains were routinely cultured in Luria broth to which ampicillin (100 $\mu g\ ml^{-1}$) was added when required. Electroporation was performed as previously described[27].

Construction of plasmids for expression of HBcAg and HPV-16 E7 epitopes

Plasmid pTetnir15 directs the expression of tetanus toxin fragment C from the *nirB* promoter[28]. A modified version of pTetnir15, which contained an additional *Nco*1 site located at the fragment C gene translation initiation codon, was used in this study (Dr J. Li, unpublished). The DNA fragment encoding fragment C was excised by cleavage with restriction enzymes *Nco*1 and *Ava*1 and replaced with the HBcAg gene. Two oligonucleotides, 5066 (5' ACAGGAAACAGTCATGA ATTC3') and 5067 (5'TACCCGGGCTAACATTGAG ATTCCCTAGATTGAGAT3'), representing sequences at the 5' and 3' ends of the gene, respectively, were used for PCR amplification of the HBcAg gene from pPN1.0[23]. The amplified fragment was digested with *Bsp*H1 and *Ava*1 and ligated to *Nco*1–*Ava*1 digested pTetnir15.

To introduce E7 DNA sequences into pGA, complementary oligonucleotides AH71 (5'CTAGCGATGTT GTAGTGAGCACGGTCCGGTTCAGCTGACCAG CCGGACCGTCGATTTCGTCTTCG3') and AH72 (5'CTAGCGAAGACGAAATCGACGGTCCGGCTG GTCAGCTGAACCGGAACGTGCTCACTACAAC ATCG3') encoding amino acid residues 35–54 of E7 (*Table 1*) were synthesised with flanking *Nhe*1 cohesive ends. Following annealing, the oligonucleotides were ligated to *Nhe*1-digested pGA. The *Nhe*1 site in pGA is located in the region of the HBcAg gene encoding the e1 loop. Plasmid pGAE7S was isolated from the products of this ligation.

Conditions for induction of *nirB* promoter, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

Salmonella strains were grown under anaerobic conditions to induce the *nirB* promoter. Briefly, overnight bacterial cultures were diluted 100-fold in Luria broth containing 100 $\mu g\ ml^{-1}$ ampicillin and incubated at 37°C with shaking for 90 min. At the end of this incubation period, the cultures were transferred to smaller flasks, overlaid with mineral oil (PCR oil from Sigma Chemical Co., Poole, Dorset, UK) and incubated at 37°C without shaking for a further 6–20 h. Bacterial cells were then harvested and resuspended in SDS–PAGE sample buffer. These samples were heated at 100°C for 5 min and cleared by centrifugation in a bench microcentrifuge. Aliquots were analysed using SDS–PAGE and western blotting. For immunoblots, nitrocellulose

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membranes were blocked with 3% bovine serum albumin in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.6) and then incubated with the primary antibody at room temperature for 2–4 h. Either an anti-HBcAg antiserum raised in guinea pig or a mixture of anti-E7 monoclonal antibodies 6D and 4F[20] were used as primary antibodies. To avoid cross-reactivity with bacterial antigens, antibodies were diluted in 1% BSA-TBST (TBS plus 0.05% Tween 20) containing a 1 in 50 dilution of *E. coli* lysate (Biorad, Milton Keynes, Buckinghamshire, UK) and incubated at 4°C overnight or at room temperature for 1 h before use. Filters were incubated with anti-species immunoglobulins conjugated to alkaline phosphatase (Sigma) and reactive polypeptides were visualised using 5-bromo-4chloro-3-indolyl phosphate, *p*-toluidine salt (BCIP, Sigma) and nitro blue tetrozolium (OPD, Sigma) as substrate.

Oral and intravenous infection of mice and enumeration of viable *Salmonella* in mouse organs

Six to eight week-old female mice, bred by B and K laboratories (Scunthorpe, Humberside, UK), were used in this study. For immunisations, *Salmonella* strains grown as static cultures in Luria broth overnight were harvested and resuspended in phosphate-buffered saline (pH 7.2) (PBS). For oral immunisations 200 μl of cell suspensions containing approximately 1×10^{10} colony forming units (c.f.u.) was administered to each mouse using a gavage needle. Between 1×10^5 and 1×10^6 c.f.u. were injected into a tail vein for intravenous (i/v) immunisations. Bacterial viability was determined by counting colony forming units on Luria agar. Groups of four orally immunised Balb/c mice were sacrificed on days 7 and 14 after immunisation. Spleens, livers, mesenteric lymph nodes and Peyer's patches were removed and homogenised as described[19]. Viable bacteria present on each homogenate were counted on Luria agar with or without ampicillin.

Immunisation schedules

For analysis of antibody responses upon intravenous immunisation 4–5 mice from strains Balb/c, C57Bl/6, B10, B10.BR or B10.D2 were immunised intravenously as described above with bacterial strains BRD969 or BRD974. A booster immunisation was administered on day 49 after the first immunisation. For studies on antibody responses upon oral immunisation, groups of 4–6 mice from strains B10, C57Bl/6 or Balb/c were immunised orally as described above on days 0 and 56. Serum samples were obtained from vaccinated mice at different times after immunisation. For lymphocyte proliferation studies, mice from strains Balb/c, C57Bl/6 or B10 were immunised orally with BRD509, BRD969 or BRD974 as described. On different dates after immunisation groups of three mice were culled and splenectomised.

Measurement of antibody responses by ELISA

Mice from different strains immunised orally or intravenously with *Salmonella* were bled from the tail at 2-week intervals. Sera were analysed in ELISA against synthetic peptide P938[12] (*Table 1*) recombinant HBcAg or a purified glutathione-*S*-transferase-E7 fusion protein (GST-E7, kind gift of Dr Germain Fernando, University of Queensland, Australia). HBcAg was prepared from *E. coli* lysates using sucrose gradients[20]. Nunc maxisorb plates were coated for 1 h at 37°C and then overnight at 4°C, with 50 μl per well of a solution of either 2 μg ml^{-1} of HBcAg in PBS, 20 μg ml^{-1} of P938 in carbonate/bicarbonate buffer pH 9.6 or 50 μg ml^{-1} of GST-E7 in PBS. After blocking with 1% skimmed milk in PBST (PBS with 0.05% Tween 20), plates were incubated with appropriate dilutions of mouse sera for 2 h at 37°C. Anti-mouse IgG- or IgA-biotin conjugates (Sigma) were used as second antibody followed by streptavidin–horseradish peroxidase conjugate (Sigma). The reaction was visualised with *O*-phenilen diamine (Sigma). Finally, optical densities at 490 nm were determined. A similar protocol was followed for ELISA assessing IgG subtype titres against P938 or GST-E7. Sera from groups of mice immunised with BRD969 or BRD974 were pooled together for titration. Titres were defined as the lowest dilution of sera to give an $O.D._{492}$ reading 0.05 unit above that of the preimmune sera. Monoclonal antibodies against different mouse IgG subtypes conjugated to biotin were obtained from PharMingen (Hull, Humberside, UK).

Measurement of intestinal IgA

B10 mice were immunised orally with *Salmonella* recombinant strains. On day 56 after the immunisation, mice were culled and their small intestines excised. Intestines were washed through with 2.5 ml of ice-cold PBS. Lavages from 4 mice per group were pooled and processed as described[21]. The total IgA present on the samples was determined by a sandwich-type ELISA with plates coated with anti-mouse IgA antibody. A solution containing a known amount of IgA was used as standard[22]. Twofold dilutions of the lavages were then assayed in ELISA using P938, GST-E7 or HBcAg as above. An ELISA unit (EU) was defined as the dilution of lavage giving an optical density of 0.05. Lavages from mice immunised with a control *Salmonella* strain were used as blanks.

Lymphocyte proliferation assays

Mice immunised orally with *Salmonella* were sacrificed on different days after immunisation and splenectomized. Single cell suspension were made from pooled spleens of four mice per group. Mice to be used as a positive control were immunised with 50 μg of peptide 8Q in complete Freund's adjuvant at the base of the tail. In this case, cell suspensions were prepared from draining lymph nodes. Standard 5-day proliferation assays were carried out, as described[14]. Different dilutions of the following recall antigens were used: HBcAg, peptide 8Q (*Table 1*) and heat-inactivated *S. typhimurium* BRD509[12]. Controls included cells stimulated with either medium, concanavalin A (Sigma) or an irrelevant peptide (derived from a non-antigenic region of E7). Cellular proliferation was measured by incorporation of ^{3}H-thymidine during the last 16 h of culture. Stimulation indexes were defined as the ratio of c.p.m. of stimulated and media-only cultures.

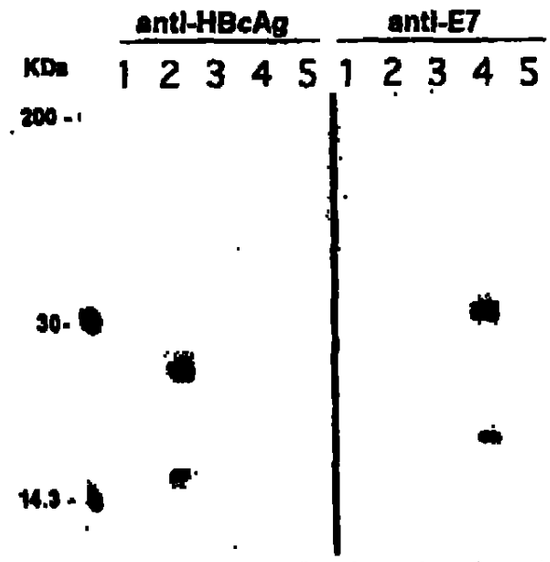

Figure 1 Western blot of *Salmonella* strains growing under aerobic or anaerobic conditions. Lane 1, BRD509, anaerobic; lane 2, BRD969 anaerobic; lane 3, BRD969 aerobic; lane 4, BRD974 anaerobic; lane 5, BRD974 aerobic. The panel on the left was probed with a guinea pig anti-HBcAg antiserum, the one on the right was probed with a mixture of monoclonal antibodies against E7 (6D and 4F). Arrows indicate the HBcAg polypeptide (left) and the chimeric E7-HBcAg polypeptide (right)

RESULTS

Stable expression of the HBcAg and chimeric HBcAg particles in *Salmonella*

Plasmid pPN1.0[23], which directs the expression of HBcAg under control of the *tac* promoter, was found to be highly unstable in BRD509 when the strain was administered to mice (our unpublished results). To overcome this problem, two plasmids, pGA and pGAE7S, were constructed in which expression of the HBcAg was under the control of the *nirB* promoter, which is induced when bacteria enter eucaryotic cells and has been used previously to stabilise the expression of foreign antigens in *Salmonella*[27,33]. Plasmid pGA encodes the HBcAg gene and several amino acid residues from the pre-core region[23]. Plasmid pGAE7S was constructed by inserting a pair of complementary oligonucleotides, encoding the E7 peptide, into pGA. pGA and pGAE7S plasmid DNA was introduced into *S. typhimurium* BRD509 and the resultant vaccine strains were named BRD969 and BRD974, respectively. Significant amounts of HBcAg were detected in strain BRD969 following growth under anaerobic conditions. The HBcAg polypeptide could be visualised by Coomassie blue staining (data not shown) and was identified by immunoblot using polyclonal antibodies against HBcAg (*Figure 1*). BRD974 produced a slightly larger chimeric polypeptide which was detected by monoclonal antibodies against E7 epitopes (*Figure 1*). This fusion product reacted weakly with anti-HBcAg antibodies, confirming previous reports which suggested that insertions within the e1 loop disrupted the antigenicity of HBcAg[23]. Electron microscopic examination of HBcAg purified from BRD969 and BRD974 detected assembled core particles (our unpublished observation).

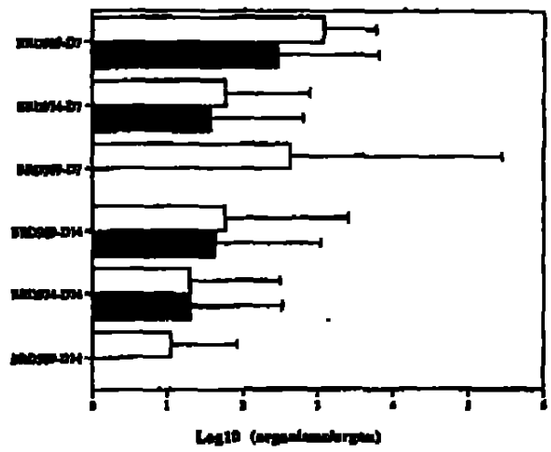

Figure 2 Stability and persistence of BRD509, BRD969 and BRD974 in the spleens of mice following oral immunisation with approximately 10^{10} bacteria. Spleens were isolated from the mice and viable counts assessed as described in Materials and Methods. Samples were taken at 7 and 14 days after immunisation. Hollow bars represent counts performed on Luria agar plates and solid bars represent counts performed on Luria agar plates containing ampicillin. The counts shown are the average of four mice sampled at each point (vertical lines represent S.D.)

In vivo stability and persistence of *S. typhimurium* strains BRD969 and BRD974

Groups of Balb/c mice were infected orally with a single dose of approximately 10^{10} BRD509, BRD969 or BRD974. On days 7 and 14 after infection mice were sacrificed to evaluate the numbers of *Salmonella* in different organs. The numbers of BRD969 and BRD974 bacteria in spleens were not significantly different from the number of BRD509 ($P>0.05$) (*Figure 2*). Almost all of the *Salmonella* recovered from organs of animals inoculated with BRD969 or BRD974 were resistant to ampicillin. Similar results were obtained when viable counts were performed in other organs including liver, mesenteric lymph nodes and Peyer's patches (data not shown). Therefore, neither pGA nor pGAE7S seemed to be significantly segregated from *Salmonella* during colonisation of the host mice.

Humoral immune response in different strains of mice following intravenous immunisation with BRD969 and BRD974

Mice from four different inbred strains were immunised i/v with 10^6 BRD969 or BRD974 and the anti-P938 and anti-HBcAg antibody responses were assessed using individual mouse serum. *Table 2* shows the results obtained with sera collected at the peak of the response, seven weeks after immunisation. All B10 (H-2^b) and 4 of 5 C57Bl/6J (H-2^b) mice mounted significant IgG responses against the E7 peptide P938 after a single immunisation with BRD974, whereas two of five B10.BR (H-2^k) and B10.D2 (H-2^d) mice and only one of five Balb/c (H-2^d) mice responded. Boosting did not detectably influence these results. All mice immunised with BRD969 or BRD974 sero-converted to HBcAg after a single i/v dose. However, levels of anti-HBcAg antibodies were higher in mice that received BRD969 compared to BRD974 (data not shown).

Table 2 Antibody to P938 in mice immunised i/v with recombinant *Salmonella* strains

Murine strain	Mean ELISA reading (S.D.) in mice immunised with strain	
	BRD 969	BRD 974
C57Bl/6	0.204 (0.026)	0.807 (0.327)
B10	0.178 (0.007)	0.417 (0.213)
B10.BR	0.291 (0.144)	0.465 (0.320)
B10.D2	0.109 (0.094)	0.119 (0.110)
BALB/c	0.059 (0.013)	0.079 (0.009)

Humoral IgG response in mice following oral immunisation with BRD969 or BRD974

B10 mice were immunised orally with 10^{10} BRD969 or BRD974 bacteria and the kinetics of the IgG antibody response against E7 and HBcAg were analysed. All mice immunised orally with BRD974 produced antibodies that recognised P938 in their sera by day 42 after the boost (*Figure 3A*). Most of the mice had high anti-P938 antibody levels after the first immunisation. Sera from mice immunised orally or i/v with BRD974 but not BRD969 recognised the GST-E7 fusion protein (*Figure 4*). Anti-P938 IgG responses induced by oral immunisation with BRD974 were mainly IgG2a and IgG2b, with lower levels of IgG1 and IgG3. I/v immunisation induced predominantly IgG2a and IgG2b. Neither IgG1 nor IgG3 anti-P938 antibody responses were detected in mice immunised i/v (*Table 3*).

In mice vaccinated orally with BRD969, high levels of anti-HBcAg humoral IgG were detected after the first immunisation, whereas there was no detectable anti-P938 response (*Figure 3A,B*). The anti-HBcAg response in mice immunised with BRD974 followed a slightly different pattern to BRD969-immunised mice. There was a steady increase in the IgG anti-HBcAg levels from day 21 to 49 after the first immunisation but the highest levels of anti-HBcAg antibody were achieved after boosting.

Humoral and secretory IgA responses in mice immunised with BRD969 or BRD974

Anti-GSTE7 and anti-HBcAg humoral IgA antibodies were detected in the serum of mice orally, but not i/v, immunised with BRD974 (*Figure 5*), whereas oral but not i/v immunisation with BRD969 elicited IgA antibodies against HBcAg only. Mice immunised orally with BRD969 or BRD974 developed strong intestinal IgA responses against HBcAg (*Figure 6*). A slight but reproducible anti-E7 intestinal IgA response was detected in BRD974 immunised mice. This response was detected using either P938 or GST-E7 as target antigen.

Cellular immune responses in mice immunised with BRD969 or BRD974

Figure 7 shows the results obtained in lymphocyte proliferation studies carried out with splenocyte populations from C57Bl/6 mice immunised orally with BRD509, BRD969 or BRD974. Similar results were obtained at different time points after immunisation or when B10 or Balb/c mice were used (not shown). Heat-inactivated *Salmonella* preparations were able to stimulate vigorous proliferation of spleen cells from the animals immunised with BRD969, BRD974 and BRD509 but not peptide 8Q (*Figure 7*). Purified HBcAg induced proliferative responses in spleen cells not only from mice immunised with BRD969 or BRD974 but also from BRD509. This was probably due to contaminating proteins trapped in the recombinant HBcAg cores that cross-reacted with *Salmonella*. Peptide 8Q was able to induce *in vitro* proliferation of spleen cells from mice primed with the same peptide, but failed to do the same in cells from mice immunised with BRD974. Similar results were obtained when splenocytes from C57Bl/6 or B10 mice were assayed on different days after two *Salmonella* immunisations (not shown).

DISCUSSION

Here we describe the construction and immunological characterisation of *S. typhimurium* vaccine strains expressing HBcAg or HBcAg harbouring a HPV-16 E7-derived peptide. We demonstrate the stability and persistence of these strains *in vivo* and show that the strains can induce anti-HBcAg and anti-E7 immune responses in a mouse model. Initial experiments utilising the *tac* promoter to drive HBcAg expression were unsuccessful as vaccine strains were found to be highly unstable *in vivo* (Londoño, unpublished observation). This problem was overcome by using the *nirB* promoter, which we have shown to be inducible within macrophages and other eucaryotic cells[27,32]. The levels of persistence of the recombinant salmonella within the host was not significantly affected by the presence of plasmids pGA or pGAE78. We detected viable, predominantly ampicillin-resistant salmonellae in the spleens and other tissues of mice orally immunised with strains BRD969 or BRD974. Previous studies utilising the *tac* promoter to express core particles in *Salmonella* either did not investigate stability or successfully utilised a system suitable for *in vivo* plasmid selection[34,35].

BRD974 was able to induce serum antibodies against E7 epitopes in different inbred mouse strains. Characterisation of the anti-P938 antibody response showed a strong bias towards the production of IgG2a at the expense of IgG1 indicating a Th1-type of helper response. This coincides with other studies in which a predominantly Th1-type response has been induced by *Salmonella* vaccination[36]. This is potentially very important as non-living, parenteral immunisation is apparently biased in favour of a Th2 response[13,20,37].

Oral immunisation with BRD974 was able to induce an anti-E7 and anti-HBcAg IgA response in the intestines of these mice. This is an important observation since HPV infections may occur through epithelial surfaces of the urogenital tract and induction of anti-HPV local responses could be critical in protection. We did not test mice for local IgA responses in their genital tracts. However, evidence for a common mucosal immune system suggests that oral immunisation may be a realistic route by which to prime other mucosal surfaces[38].

Given the successful induction of humoral and secretory responses against E7 by oral immunisation of mice with BRD974, it was surprising that E7-specific T-cell proliferative responses were not detectable in these animals (*Figure 7*). However, T-cell immunogenicity of recombinant proteins expressed in live

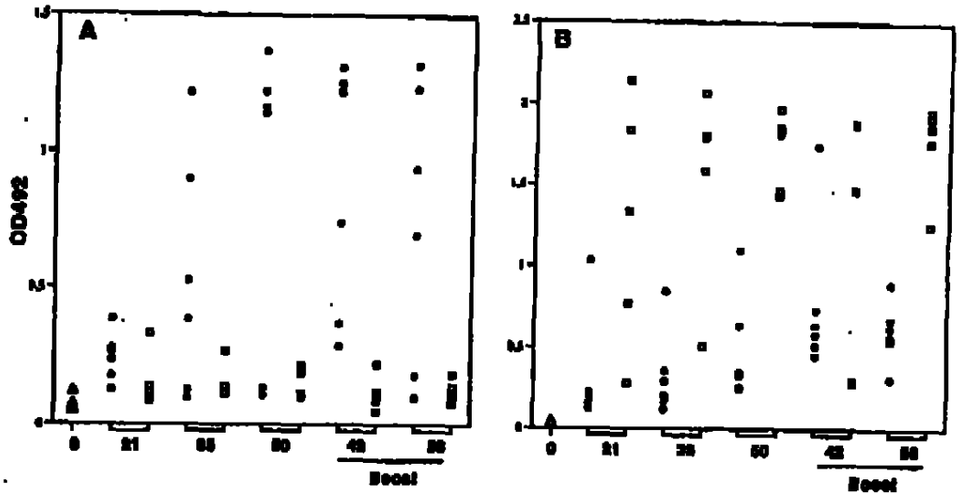

Figure 3 The kinetics of the antibody response against (A) P33B or (B) HBcAg in the serum of mice orally immunised with approximately 10^{10} BRD969 (open squares) or BRD974 (solid dots) on days 0 and 52. Preimmune sera (open triangles) are also shown. Individual serum samples were diluted 1:200 (for anti-HBcAg ELISA) or 1:50 (for anti-P33B ELISA) and tested as described in Materials and Methods

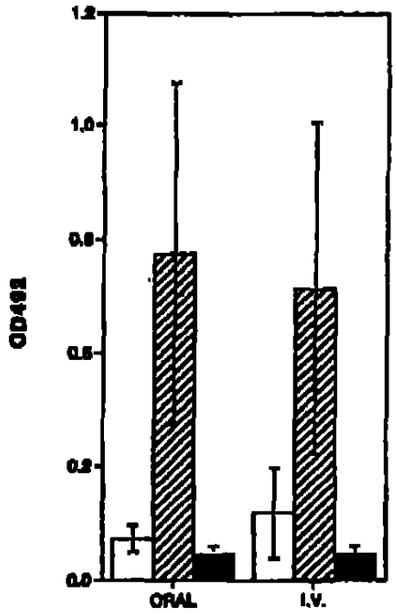

Figure 4 Level of IgG anti-GST-E7 antibodies in the serum of mice orally or intravenously immunised with BRD969 (hollow bars) or BRD974 (striped bars). Levels of anti-GST-E7 antibodies in the pre-immune serum, taken on day prior to immunisation, are shown by the solid bar. Individual mice sera were tested in ELISA at 1:100 dilution as described in Materials and Methods. Results are expressed as an average, with the standard deviation represented by the error bars

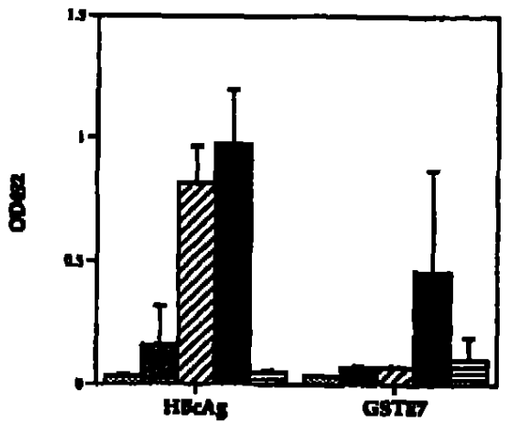

Figure 5 Serum IgA response against GST-E7 and HBcAg in mice orally immunised with BRD509 (densely stippled bars), BRD969 (slanting hatched bars) or BRD974 (solid bars) or intravenously immunised with BRD974 (striped bars) or BRD969 (dotted bars). Antibody levels were determined using individual mice serum (1:50 dilution), as described in Materials and Methods, and are the average of 4 or 5 mice. Error bars represent the standard deviations

Table 3 Serum IgG subtype anti-P33B titres after a single immunisation with BRD974

IgG subclass	Oral immunisation	I/v immunisation
IgG1	<50	<50
IgG2a	450	450
IgG2b	450	50
IgG3	<50	<50

Salmonella vaccine strains have been varied. For example, mice or swine immunised orally with *S. typhimurium* expressing the 31-kDA protein of *Brucella abortus* exhibited serum and mucosal responses against the antigen, but neither delayed-type sensitivity nor *in vitro* proliferative responses against the protein were detected[39,40]. On the other hand, Brett and colleagues[41] showed the influenza virus nucleocapsid antigen elicited a T-cell response with the same fine specificity when delivered by either a *Salmonella aro* mutant or as a purified protein emulsified with adjuvant. One possible explanation is that the recombinant proteins would compete with native *Salmonella* antigens for immunodominance in the host. The final outcome may depend

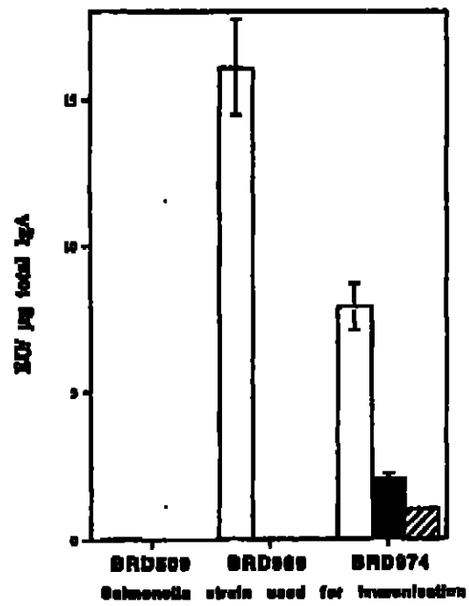

Figure 6 Intestinal IgA levels against E7 or HBcAg in mice orally immunised with *Salmonella* recombinants. Levels of anti-GST-E7 (solid bars), anti-P93B (striped bars) or anti-HBcAg (hollow bars) IgA were determined as described in Materials and Methods

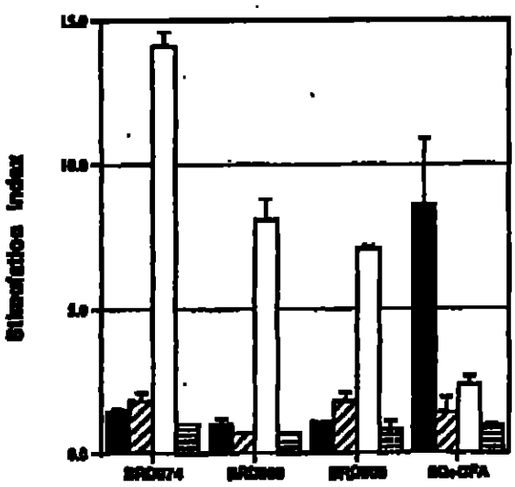

Figure 7 Spleen cell proliferation in mice orally immunised with BRD909, BRD969 or BRD974 or subcutaneously with peptide SQ. Mice were sacrificed 42 days after immunisation with the recombinant *Salmonella* strain or 12 days after immunisation with peptide SQ. Recall antigens were SQ (3 μg ml^{-1}, solid bars), HBcAg (250 ng ml^{-1}, slanting hatched bars), heat-inactivated *Salmonella* (1.5×10^{8} c.f.u. ml^{-1}, hollow bars) and control peptide (3 μg ml^{-1}, horizontal striped bars). Values are arithmetic means of quadruplicate wells and error bars represent standard deviations

on the relative ability of their degradation products to bind the appropriate MHC molecules. The strong binders would dominate the T-cell responses and provide help for the production of antibodies. Macrophages are known to process *Salmonella* antigens using classical and non classical pathways[42]. This may influence the presentation of the particles to T cells, compared to the processing of purified particles. However, our understanding of the processing and presentation of bacterial antigen by salmonellae-infected macrophages is still very limited.

The *Salmonella* strain used in this study can induce *in vivo* CTL responses against heterologous antigens such as ovalbumin and we are engaged in studying the possibility of inducing CTL responses against HPV-16 infected cells using a strain similar to BRD974 which expresses an E7-derived CTL epitope. The availability of *Salmonella typhi* strains suitable for vaccination of humans also means that the clinical evaluation of these constructs can be undertaken[43].

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Reivindicaciones

1. Una composición de vacuna que comprende un antígeno de superficie de Hepatitis B y un antígeno del virus de papiloma humano (HPV), junto con un adyuvante el cual es un estimulador preferencial de respuesta de célula TH1, en donde la composición de la vacuna no comprende un antígeno viral de herpes simples, en donde la composición es indicada para el tratamiento o profilaxis de infecciones del virus de papiloma humano e infecciones de hepatitis B, y en donde el antígeno PPV comprende la proteína cápsida mayor L.1 de HPV de HPV 16 y/o HPV 18, o una fusión de proteína D de *Haemophilus influenza B* con E7 de HPV 16.

2. Una composición de vacuna según la reivindicación 1 que adicionalmente comprende un vehículo.

3. Una composición de vacuna según la reivindicación 1 ó 2, en la cual el estimulador preferencial de la respuesta de la célula TH1 es seleccionado del grupo de adyuvantes que comprenden; 3D-MPL, 3D-MPL, en donde el tamaño de las partículas de 3D-MPL es preferiblemente de aproximadamente o inferior a 100 nm, QS21, una mezcla de QS21 y colesterol y un oligonucleótido CpG.

4. Una composición de vacuna según la reivindicación 3, en la cual el estimulador preferencial de la respuesta de célula TH1 es 3D-MPL.

5. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 4, en la cual el antígeno del virus Epstein Barr (EBV) está adicionalmente presente.

6. Una composición de vacuna según se define en la reivindicación 5, en la cual el antígeno EBV es gp 350.

7. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 4, en la cual un antígeno de hepatitis A está adicionalmente presente.

8. Una composición de vacuna según la reivindicación 7, en la cual el antígeno de *Hepatitis A viral* (HAV) se deriva de la cepa HM-175.

9. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 8, en la cual el vehículo es seleccionado del grupo compuesto por hidróxido de aluminio, fosfato de aluminio y tocoferol en una emulsión aceite en agua.

10. Una composición de vacuna según las reivindicaciones 1 a 9, que adicionalmente comprende el antígeno de *Varicella Zoster viral* (VZV).

11. Una composición de vacuna según la reivindicación 10, en la cual el antígeno VZV es gpI.

12. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 11, que adicionalmente comprende un antígeno de citomegalovirus humano (HCMV).

13. Una composición de vacuna según la reivindicación 12, en la cual el antígeno HCMV es gB685* o pp65.

14. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 13, que adicionalmente comprende un antígeno *Toxoplasma gondii.*

15. Una composición de vacuna según la reivindicación 14, en la cual el antígeno *Toxoplasma gondii* es SAG1 O TG34.

16. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 4, que comprende L2, E6, E7, proteína D-E6, proteína D-E7 o L2-E7 de HPV y opcionalmente, en adición, uno o más de HSV-2 gDt; EBVgp 350; VZVgpI; cepa inactivada de HAV HM-175; gB685** o pp65 de HCMV y antígenos SAG1 o TG34 de *Toxoplasma gondii.*

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCI
DIVISIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Doctor
GERMAN CASTILLO GRAU
Smithkline Beecham Biologicals
(Apoderado)

ASUNTO

Radicación 00-66606
Trámite 002
Evento 001
Actuación 430
Oficio 532
Folios

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

Documentos estudiados:

- El capítulo descriptivo (Folios: 4 a 59)
- Las nuevas reivindicaciones 1 a 16 (folios 148 a 149)que reemplazan el capítulo descriptivo encontrado en folios: 60 a 63
- No se aceptan las reivindicaciones1, 6 a 7 y 9 a 17. Puesto que la materia reclamada en ellas no se encuentra enteramente soportada por la descripción. El mencionar que se van a emplear ciertos antígenos en una composición dada no quiere decir que esto constituya una invención, más bien parece expresar una intención hacia futuro por parte del solicitante. Tras la lectura de toda la descripción no se encontró que las formulaciones reclamadas en las cláusulas en mención estén definidas a través de sus componentes ni de la proporción de éstos en la mezcla. Así mismo, ninguna de las formulaciones de las realizaciones preferenciales empleadas en los ensayos de inmunogenicidad (figuras 1 a 9) contienen antígenos tipo EBV(gp30), HAV (HM-175), VZV (gpI), HCMV (gB685 o pp65), SAG1 o TG34, o una fusión de proteína D de Haemophilus influenzae B con E7 de HPV 16.
- Se acepta la prioridad reivindicada GB Nº 9921146,8. de 7 de septiembre de 1999
- Respuesta a los conceptos técnicos 1347 y 1350 radicada el 5 de diciembre de 2005

Objeto de Invención:

El objeto de la presente solicitud se refiere a una composición de vacuna que comprende un antígeno del virus de hepatitis B (HBV), un antígeno de papiloma humano (HPV) más un coadyuvante estimulador Th1 y un vehículo. En donde el antígeno de hepatitis es un antígeno de superficie de hepatitis B, el antígeno HPV puede es L1 L1 de HPV16 o HPV18, o una fusión de proteína D de Haemophilus influenzae B con E7 de HPV16 , el adyuvante estimulador Th1 es 3DMPL y el vehículo se selecciona entre $Al(OH)_3$, $AlPO_4$, tocoferol o emulsión O/W Las composiciones pueden contener además 1 o más antígenos derivados de EBV, HAV, HSV, VZV, HCMV, Toxoplasma gondii.

El objeto de la solicitud definido anteriormente se clasificó en la IPC A61K 39/00, 39/29, 39/295.

Determinación del estado de la técnica:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de Patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40".

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: 7 de septiembre de 1999, se buscaron documentos del estado de la técnica relacionados, con fecha de publicación anterior a esta.

Se hizo la búsqueda de documentos relevantes en el estado de la técnica, entendiendo como estado de la técnica la definición provista en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina y las excepciones fijadas en el artículo 17 de la misma.

Se encontró como anterioridades (documentos cuya fecha de publicación es anterior a la fecha de presentación de la primera solicitud de patente sobre la cual se reivindica prioridad o de la fecha de presentación de esta solicitud) que pueden afectar la patentabilidad del objeto de la presente solicitud:

Nº	Documento	Título	Fecha de Publicación
1	WO95/17209	VACCINES	29 de junio de 1995
2	TINDLE ET AL.Virology, Volume 200, Issue 2,pag 547 a 557	Chimeric hepatitis B Core Antigen Particles Containing B- and Th-Epitopes of human Papillomavirus Type 16 E7 Protein Induced Specific Antibody and T Helper Responses in Immunized Mice	1 de mayo de 1994
3	LONDOÑO ET AL. Vaccine, Volume 14, Issue 6, pag 545-552	Immunisation of mice using Salmonella typhimurium expressing human papillomavirus type 16E7 epitopes inserted into hepatitis b virus core antigen	Abril 1996

Evaluación de los requisitos de Patentabilidad:

El Artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina el cual establece: "Los Países Miembros otorgarán Patentes para las invenciones, sean de producto o de procedimiento en todos los campos de la tecnología siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

Se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

Evaluación de Nivel Inventivo:

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".

El Nivel Inventivo se evaluó por comparación entre lo solicitado y encontrado en el estado de la técnica y que se relaciona con el objeto reivindicado y de acuerdo con la definición de nivel inventivo del Artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

Las reivindicaciones 1 a 4 de la solicitud en estudio reclaman composiciones de vacunas que comprenden un antígeno de Hepatitis B (HBV), un antígeno de papiloma humano (HPV) más un coadyuvante estimulador Th1 y un vehículo. En donde el antígeno de hepatitis es una antígeno de superficie de Hepatitis B, el antígeno HPV es L1 L1 de HPV16 o HPV18, o una fusión de proteína D de Haemophillus influenzae B con E7 de HPV16, y el adyuvante estimulador Th1 se selecciona del grupo 3DMPL, QS21, o una mezcla QS21+colesterol+CpG. La composición puede contener una proteína de fusión o una proteína truncada.

La parte de la cláusula 1 referente a la fusión de proteína D de Haemophillus influenzae B con E7 de HPV16, no encuentra suficiente sustento en la descripción, dado que se menciona únicamente como una dentro de muchas fusiones preferidas, de las cuales no se muestran datos ni ensayos concretos y por ende no pueden ser consideradas como parte sustantiva de la invención sino como una intención a futuro del solicitante.

El documento Tindle et Al, de ahora en adelante denominado D2, revela la el uso del antígeno de superficie de Hepatitis b (HBcAg) como un vehículo inmunogenico para otros epitopes y describe específicamente una partícula quimérica de Hepatitis b (HBcAg) expresando epítopes E7 de HPV. Tras la inmunización de ratones con dichas partículas se observó tanto respuesta humoral como proliferación de Células T.

Siendo que el antígeno (HBcAg) es un antígeno de superficie de hepatitis B usado ampliamente como carrier para antígenos patógenos, como también lo revela la anterioridad D3, se establece que las vacunas de las reivindicaciones 1 a 4 se diferencian de las divulgadas en D1, en el adyuvante estimulador de la respuesta Th1.

Tal como se había mencionado en el concepto técnico 1347 en la práctica es común el empleo de adyuvantes estimuladores de la respuesta Th1, tales como 3DMPL, saponinas, QS21 para potenciar la respuesta inmune a un antígeno dado. Esto debido a que la respuesta Th1 a través de interferón alfa esta asociada con respuestas protectivas frente a patógenos intracelulares. De por sí el Documento WO95/17209 ó D1, divulga en las reivindicaciónes 1 y 7 el empleo de 3DMPL y QS21 en vacunas que contienen antígenos de Hepatitis B(HBV), Papiloma virus humano (HPV), toxoplasma, citomegalovirus humano, Herpes Simplex, en forma de emulsión O/W con el objeto de obtener respuestas inmunológicas mejoradas.

Dado que en la anterioridad D1 ya había sido divulgado el uso de adyuvantes 3DMPL y QS21 en vacunas conteniendo antígenos provenientes de Hepatitis B y de Papilomavirus humano (HPV) y en D2 se habían revelado partículas quiméricas que

comprenden la combinación del antígeno de superficie de HBV con el epítope E7 de HPV , resulta obvio para una persona medianamente versada en el desarrollo de vacunas, generar composiciones de vacuna en las cuales se usan las partículas quiméricas o la combinación de antígenos ya conocidas y adicionar los adyuvantes ya recomendados en D1 para los mismos.

El solicitante afirma en su respuesta del 5 de diciembre que una persona versada en la materia no buscaría incrementar la respuesta Th1 dentro de la composición puesto que la sugerencia en D2 es el uso de adyuvantes como alhidrogel estimulantes de la respuesta Th2. Cabe mencionar que el solicitante también emplea adyuvantes de aluminio estimulantes de la respuesta Th2 en las composiciones reclamadas (reivindicación 9); ésto por que hasta el momento también eran adyuvantes ampliamente empleados en la formulación de vacunas y no porque le interesara únicamente obtener una respuesta tipo Th2. Sin embargo, un complemento obvio para sus composiciones inmunogénicas estaría en la introducción de adyuvantes estimulantes de Th1, cuyo uso y efectividad con antigenos del mismo origen de los empleados en la presente solicitud había sido divulgado previamente en D1 y en muchas composiciones de vacuna desarrolladas posteriores a la publicación de D1 en las cuales se buscaba incrementar la respuesta inmune frente a patógenos.

Aún cuando no se aceptan las reivindicaciones 5 a 8 y 10 a 16 por no estar enteramente sustentadas por la descripción, vale la pena advertir que las vacunas polivalentes que incluyen epítopes antigénicos de diferentes orígenes, son conocidas en el estado del arte. De esta forma un técnico en la materia podría de manera evidente crear proteínas fusionadas a partir de sus epítopes de interés y de antígenos de patógenos ampliamente conocidos en el estado del arte, para obtener composiciones más inmunogénicas. Así que el objeto contenido en las clausulas en mención tampoco tendría cumpliría con el requisito de nivel inventivo.

Por último los vehículos de hidróxido y fosfato de aluminio son ampliamente empleados en vacunas y en formulaciones de emulsiones tipo O/W y por ende la reivindicación 9 no sería considerada como inventiva.

La respuesta al problema técnico que se pretendía resolver, que era proveer vacunas combinadas para la prevención frente a infecciones por virus de papiloma humano (HPV) resulta derivado de la combinación de las anterioridades D1 y D2. A lo largo de la solicitad no se encuentra evidencia que la presente invención pueda representar un salto cualitativo respecto de las composiciones existentes en el estado de la técnica.

Como se observa, el objeto de esta solicitud podría resultar obvio para una persona del oficio normalmente versada en la materia y derivarse de manera evidente del estado de la técnica.

En conclusión los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

Nº	Documento	Reivindicaciones afectadas	Requisito que afecta
2.	TINDLE ET AL	1-4,	Nivel Inventivo
4	WO95/17209	2-4, 9	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad

Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista, de fecha 09 ENE. 2006

CONCEPTO TÉCNICO N°: 2089	**EXAMINADOR TÉCNICO Código N°: 202063**	**2005-12-28**