

The present invention relates to novel vaccinal preparations useful against tumours induced by papillomaviruses, and more particularly against types HPV-16, HPV-18, HPV-31, HPV-33, HPV-35.

The papillomaviruses are a group of DNA viruses. They have a protein shell and a circular DNA genome of approximately 7900 base pairs. A certain number of types of papillomaviruses, bovine (BPV), human (HPV) have been identified and the genomes of certain of them have been completely sequenced (1).

The fundamental research work conducted on these viruses has thus led to a distinction being made within their genome between an early region and a late region, by analogy with the genome of the polyoma virus and of SV40. The late region contains two reading frames L1 and L2 which code for the major components of the capsid. The early region contains at least the reading frames E1, E2, E4, E5, E6, E7 and E8. The proteins coded by the reading frames have different functions. Three of these proteins are involved in the oncogenic transformation process of infected cells. Protein E5 of BPV-1, which has great transforming capacity and which may independently transform cells *in vitro* (2), is coded by part 3' of the early region. The proteins E6 of BPV-1 and E7 of HPV-16 are coded by part 5' of the early region and are involved in the induction and maintenance of the oncogenic transformation. These two proteins apparently derive from a common ancestral gene by successive duplications of a 33 amino acid peptide (3). The transforming capacity of E7 has been demonstrated for HPV-16 and 18 (4, 5, 6). Of the other early proteins, E1 and E2 have a role in the replication and/or expression of the virus, while no function has been identified for E4 and E8.

In man, the HPV are associated with pathologies ranging from benign skin infections to warts and to malignant tumours. These viruses are highly specific to the target tissues, particularly the epidermal epithelia of the genital, oral

and respiratory tracts (7). Epidemiological data strongly suggest a role for certain strains of HPV in cancer of the uterine cervix and the pelvic passages (the most frequently fatal tumour in women). The DNA of HPV-16 and HPV-18 is found in most biopsies taken from cancerous genital cells, HPV-31, HPV-33, HPV-35, HPV-39 and HPV-45 are more rarely detected.

The pathologies associated with HPV viruses constitute a therapeutic problem due to their persistent and recurrent nature. Many approaches have already been used in the treatment of these illnesses: surgery, chemotherapy, anti-viral agents and immunotherapy (8).

In particular, European Patent application EP-A-0133123 describes a vaccination approach against papillomavirus infection which consists in preparing virus capsid proteins by genetic engineering, i.e. proteins of the structure corresponding to the late region of the virus, and using them as immunogenic agents. The means described in this document are intended to provide protection against infection by the viruses themselves and therefore *a priori* against all forms of infection likely to occur.

The aim of the present invention is more particularly to obtain active vaccines for preventive or curative treatment of malignant tumours resulting from a pre-existing HPV infection.

The present invention is based on the observation that tumour formation induced by papillomaviruses is due to the expression of the early genes of the virus.

The invention therefore provides pharmaceutical preparations characterised in that they contain as active preventive or curative constituent at least one recombinant pox virus comprising a heterologous DNA sequence coding for at least

the essential region of an early protein of a papillomavirus together with the regulating factors ensuring its expression in higher cells.

The essential region of the said protein is taken to mean that part of the protein sequence capable of inducing anti-tumoral immunity.

The vaccinal preparations according to the invention may be used for various purposes. They may be used preventively to prevent the appearance of malignant tumours, either before any infection by the virus, or even after an infection having caused benign disorders, in case other tissues are affected and transformed into cancerous tissues. They may also potentially be used curatively to cause the disappearance of an already established tumour or in addition to or instead of ablation. These uses may relate to man, but also to animals, in particular to cattle to prevent BPV virus infections. If need be, the recombinant pox virus may be used with a pharmaceutically acceptable carrier for its inoculation into man or animals.

Given the observations which have been made and mentioned above concerning the frequency of infection by HPV type 16-18-31-33-35-39 and 45 in the case of uterine cervical cancer, the invention more particularly relates to vaccinal preparations useful against HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39 and HPV-45.

It is for this reason that the invention provides a vaccinal preparation as previously defined in which the pox virus comprises at least one DNA sequence coding for a least one non-structural protein of the type 16, 18, 31, 33, 35, 39 or 45 HPV virus and in particular of the HPV-16 virus.

As previously mentioned, the structure of viruses of the papilloma family, in particular BPV-1 and HPV, is such that

there are at least 7 reading frames likely to correspond to protein functions which are highly specific to the mechanisms governing the action of the virus and maintaining the viral genome. It is for this reason that it may be worthwhile making pharmaceutical preparations which simultaneously express several proteins in the human body. This may be achieved either with several pox viruses each expressing a given protein or with one pox virus containing several sequences of heterologous DNA corresponding to the selected proteins.

Naturally, the recombinant pox virus will include all the factors necessary for the expression of the proteins in higher cells, i.e. generally a gene transcription promoter and a region for the initiation of translation in the host cell; the promoter will preferably be the promoter for a gene of the pox virus used.

Among all the conceivable pox viruses, it is preferably the vaccinia virus which is chosen, because it has already been established that the antigens expressed by the recombinant vaccinia virus are correctly presented at the surface of the infected cells and allow a cellular type immune response to be induced. The circumstances are therefore particularly favourable since it is known that the elimination of tumour cells involves cellular immunity.

When the pox virus is the vaccinia virus, the promoter used will preferably be the promoter for the gene of the 7.5 K protein of the vaccinia virus. The promoter/coding sequence combination will be inserted into a non-essential region of the virus; for example, in the case of the vaccinia virus, the thymidine kinase (TK) gene, which will allow the ready selection of TK<sup>-</sup> recombinant viruses.

In general, the living virus is inoculated into man or animals. It is, however, also conceivable to inject the

killed recombinant virus into man or animals, which virus presents on its surface the selected proteins, or the proteins purified from cell cultures infected with the recombinant vaccinia viruses.

The pharmaceutical preparations according to the invention may be produced according to processes known in the vaccines sector, and the dosages which may be administered may vary over a wide range. The dosages will depend, in particular, on the patient's condition and other parameters to be assessed by the practitioner.

The invention will be illustrated with the following examples which, on the one hand, describe the results of vaccination in animals which were injected with cells transformed by the bovine papilloma virus (BPV-1) and, on the other hand, the results of vaccination in animals which were injected with cells transformed by the human papilloma virus (HPV-16). In particular, the cloning and sequencing of the DNA corresponding to the reading frames for proteins E5, E6, E7 from HPV-16 are described, as are the results achieved by vaccination with recombinant viruses expressing these proteins.

The detailed description of the invention is accompanied by figures 1 to 7, which show:

- figure 1 shows the SDS-PAGE gel of the immunoprecipitates of the products of reading frames E1, E2, E5 and E6 of the early region of the genome of bovine papillomavirus (BPV-1).
- figure 2 shows the latency period for the development of tumours in animals vaccinated with the recombinant vaccinia viruses expressing the products of reading frames E1, E2, E5, E6 or E7 of bovine papillomavirus BPV-1 (VVbE1, VVbE2, VVbE5,

VVbE6 and VVbE7) or a control recombinant vaccinia virus, VV-0 (not expressing a BPV-1 protein) and tested with FR3T3 cells transformed by BPV-1 (FR3T3 BPV-1-6).

- figure 3 shows the latency period for the development of tumours in animals vaccinated with the recombinant vaccinia viruses expressing products of reading frames E5, E6 and E7 of bovine papillomavirus (BPV-1) (VVbE5, VVbE6, VVbE7) or a combination VVbE5-VVbE6 or VVbE5-VVbE7 or a control recombinant vaccinia virus VV-0 (not expressing a BPV-1 protein) and tested with FR3T3 cells transformed by BPV-1 (FR3T3-BPV-1-3).
- figure 4 is a schematic representation of the structure of bacteriophage M13 E7/E6 (HPV-16).
- figure 5 is a schematic representation of the structure of bacteriophage M13 E5 (HPV-16).
- figure 6 shows the complementary DNA structure of reading frame E5 (HPV-16).
- figure 7 shows the SDS-PAGE gel of the immunoprecipitates of the products of reading frames E6 and E7 of the early region of the genome of human papillomavirus HPV-16.
- figure 8 shows the development in size of tumours in animals vaccinated with the recombinant vaccinia virus expressing the product of reading frame E6 of human papillomavirus HPV-16 (VVhE6) and tested with primary rat cells transformed by HPV-16 and a ras oncogene.

- figure 9 shows the development in size of tumours in animals vaccinated with the recombinant vaccinia virus expressing the product of reading frame E7 of human papillomavirus HPV-16 (VVhE7) and tested with primary rat cells transformed by HPV-16 and a ras oncogene.

Example 1: Inhibition of development of tumours induced by bovine papilloma virus (BPV-1) by vaccination with recombinant vaccinia viruses expressing the early proteins of BPV-1.

a) Construction of vaccinia viruses expressing the early proteins of BPV-1

Plasmid pM69 (9) contains the early region of the BPV-1 genome inserted as a HindIII-BamHI fragment in the HindIII and BamHI sites of plasmid pML2 (9). Sub-fragments containing the reading frames E1, E2, E5, E6 and E7 are excised by digestion with restriction enzymes (see table I) and introduced into bacteriophages M13TG130 or M13TG131 (10), then subjected to localised oligonucleotide-directed mutagenesis before being transferred in plasmid pTG186poly (11). These stages are summarised in table I.

Key to table I:

- [a]: the underlined nucleotides indicate the bases which do not match the BPV-1 parental sequence
- [b]: the translation initiation codon and the restriction site used in the cloning are underlined.
- [c]: it was not possible to introduce the EcoRI-BamHI fragment of plasmid pM69 containing the E5 reading frame into bacteriophage M13TG131 in the desired orientation: the clones are obtained in the inverted orientation in which two independent plasmid fragments

are cloned; mutagenesis by oligonucleotide does therefore correspond to the other DNA strand.

[k]: end produced by digestion by a restriction enzyme followed by treatment with the Klenow fragment of DNA polymerase I from *E. coli* before cloning;  
na: not applicable

**Table I:** Cloning and mutagenesis of sub-fragments of BPV-1 genome for their transfer into vaccinia viruses.

| BPV-1              |                      | Mutagenesis                                     | Cloning                |
|--------------------|----------------------|-------------------------------------------------|------------------------|
| • reading frame    | • vector site        | • sequence (5'-3') of oligonucleotides used [a] | • fragment             |
| • fragment         |                      | • sequences at translation initiation site [b]  | • vector site          |
| E1                 | M13TG130             | na                                              | <u>BamHI-SstI</u>      |
| <u>NruI-AvrII</u>  | <u>BamHI[k]-XbaI</u> | (TCGCGAGCGTCATGG)                               | pTG186poly             |
|                    |                      |                                                 | <u>BamHI-SstI</u>      |
| E2                 | M13TG131             | GAGGAGGATCCTGAAGAGGA                            | <u>BamHI</u>           |
| <u>EcoRI-SpeI</u>  | <u>EcoRI-XbaI</u>    | (GGATCCTGAAGAGGATGG)                            | pTG186poly             |
|                    |                      |                                                 | <u>BamHI</u>           |
| E5                 | M13TG131             | AGATTTGCCATAGTCGACCAGTCA[c]                     | <u>SalI-BamHI</u>      |
| <u>EcoRI-BamHI</u> | <u>EcoRI-BamHI</u>   | (GTCGACTATGG)                                   | pTG186poly             |
|                    |                      |                                                 | <u>SalI-BamHI</u>      |
| E6                 | M13TG130             | CAGACCCCGGATCCAACATGGACCT                       | <u>BamHI-XmaIII[k]</u> |
| <u>HpaI-SmaI</u>   | <u>SmaI</u>          | (GGATCCAACATGG)                                 | pTG186poly             |
|                    |                      |                                                 | <u>BamHI-SmaI</u>      |
| E7                 | M13TG130             | TGCTACGACTCGAGCAAACATGGTTCA                     | <u>XhoI-EcoRI</u>      |
| <u>HpaI-SmaI</u>   | <u>SmaI</u>          | (CTCGAGCAAACATGG)                               | pTG186poly             |
|                    |                      |                                                 | <u>SalI-EcoRI</u>      |

The localised oligonucleotide-directed mutagenesis allows a translation initiation consensus sequence to be introduced at the initiation codon so as to ensure correct expression after transfer into the vaccinia virus. In each case, the synthesis oligonucleotide introduces a single restriction site just before the sequence determining the start of translation.

In the case of reading frame E1, it is not necessary to perform the localised mutagenesis due to the presence of a restriction site in the BPV-1 genome (site NruI in

position -11 in relation to the start ATG) and of a quasi consensus sequence of the translation initiation region (GCGTCATGG): the nucleotide G in position -3 is nearly as effective as A in initiating translation.

Some primary chicken embryo cells are cultivated at 37°C in a MEM (Gibco) medium supplemented with 10% foetal calf serum. The cells are simultaneously subjected to infection with a heat-sensitive vaccinia virus and to transfection by the expression/transfer vectors bearing the inserted BPV-1 segments and by DNA from the wild type vaccinia virus. After selection, vaccinia recombinants in which expression of the inserted sequence is under the control of the 7.5 K promoter of the vaccinia virus are isolated using customary techniques.

The recombinant vaccinia viruses (VV) expressing the early proteins of BPV-1 E1, E2, E5, E6 and E7 are respectively named VVbE1, VVbE2, VVbE5, VVbE6 and VVbE7.

b) Expression of E1, E2, E5, E6 and E7 in cells infected by the recombinant viruses

Some BHK-21 cells on Oulbecco MEM.BME modified Eagle medium (GIBCO) supplemented with 10% foetal calf serum (GIBCO) are infected with one of the six recombinant viruses with a plurality of approximately 20 plaque forming units (pfu) per cell.

After one hour at 37°C, fresh medium is added and the cells are incubated for two hours. The medium is drawn off, the cells washed once. Some MEM.BME medium without methionine and/or cysteine and supplemented with 5% dialysed foetal calf serum is then added. Labelling is performed with 0.5-1 mCi/ml of L-methionine-35S and/or L-cysteine-35S (Amersham) for 3 hours at 37°C. The cells labelled in this manners are rinsed twice with 20 mM tris-HCl pH 7.2;

150 mM NaCl (TS buffer) containing aprotinin (1 UI/ml; Biosys, France), collected by scraping up and rinsed by centrifuging twice in STE buffer (20 mM tris-HCl pH 7.2; 150 mM NaCl; 1 mM Na<sub>2</sub>EDTA; 1% aprotinin). The cells are lysed in RIPA buffer [50 mM tris-HCl; pH 7.4; 150 mM NaCl; 1 mM Na<sub>2</sub>EDTA; 1% Triton X-100; 1% NaDeoxycholate; 0.1% SDS] or fractionated in order to determine the subcellular location of the BPV-1 proteins.

Immunoprecipitation and SDS-PAGE are performed in accordance with the procedure described by Davis (12). Polyclonal rabbit antisera directed against the bacterial fusion proteins corresponding to reading frames E1, E2, E5, E6 and E7 are obtained using the conventional method known to the person skilled in the art, see for example references (13) and (14).

The cells infected with VVbE1 produce a 69 kDa nuclear protein which is specifically recognised by the anti-E1 antiserum (figure 1, band 1, band 2 relates to immunoprecipitation with a non-immune serum). The cells infected with VVbE2 contain a high level of a 48 kDa polypeptide having cross-reactivity with E2 and corresponding to the major transactivation protein coded by reading frame E2 of BPV-1 (figure 1, band 3 and control, band 4). This polypeptide is detected in all the cellular sub-fractions examined, in accordance with what has been described for protein E2 of cells transformed by BPV-1 (15). VVbE6 codes for a 15.5 kDa protein which is specifically precipitated by anti-E6 serum (figure 1, band 6 and control band 7) and over 50% of which is located in the cytoplasmic fraction, as for protein E6 of cells transformed by BPV-1 (16). Cells infected with VVbE5 produce a 7.5 kDa polypeptide associated with the cell membranes (figure 1, band 7 and control, band 8), the characteristic location of protein E5 of BPV-1 (17). Immunoprecipitation tests with anti-E7 antiserum do not exhibit cross-reactivity.

Nevertheless, analysis of the expression vectors reveals the presence of reading frame E7 in a correct orientation and we assume that E7 is actually produced but for an unknown reason is not precipitated by the available antiserum.

c) Vaccination against tumour cells induced by BPV-1

Groups of 4 week old female (Fischer) rats are intradermally or intraperitoneally inoculated with the various recombinant viruses (at a dosage of  $10^8$  pfu in 100  $\mu$ l). A booster injection with the same dose is administered after 12 days and a test inoculation is administered between the 16th and 17th day with 3T3 cells from syngenic lines of Fischer rats (FR3T3) transformed by BPV-1 in accordance with the protocol described in references (9, 18) and named FR3T3-BPV-1-6. To this end,  $2 \times 10^4$  cells are injected subcutaneously in a volume of 200  $\mu$ l of MEM.BME medium without serum.

The results are shown in figure 2:

- all the control animals vaccinated with VV-0 develop tumours after an average latency period of 43 days.
- animals vaccinated with VVbE1 or VVbE2 exhibit no change in tumour development.
- animals vaccinated with VVbE5, VVbE6 and VVbE7 exhibit a significant delay in the appearance of tumours. Moreover, in several cases no tumours develop (> 250 days after test), which are the dots shown in the box at the top of the figure.

These experiments are repeated with the test inoculation being performed with FR3T3 line cells transformed by BPV-1 in accordance with the protocol described in references (9, 18) and named FR3T3-BPV-1-3. Since VVbE1 and VVbE2 have

no effect, only VVbE5, E6 and E7 are tested and two groups of rats are inoculated with two recombinant viruses simultaneously, either VVbE5 and VVbE7 or VVbE5 and VVbE6 in order to detect any possible cumulative effect of these antigens. Figure 3 confirms the beneficial effect of vaccination with VVbE5 and VVbE7 (the dots in the box at the top of the figure correspond to animals which do not develop any tumours), while VVbE6 remains without any noticeable effect in this situation. Moreover, there appears to be no marked cumulative effect when VVbE5 and VVbE7 are associated.

Example 2:        Cloning of genome of HPV-16.

Cells of the line CaSki (ATCC 1550) contain the genome of the HPV-16 virus incorporated into a chromosome. Starting from ten 75 cm<sup>3</sup> vials of semi-confluent CaSki cells, the total genomic DNA is purified in the following manner: the cells are collected by scraping, centrifuged and washed and then taken up in 15 ml of TE buffer [tris 10 mM pH 7.5; EDTA 1 mM] + 0.5% SDS. After treatment with proteinase K (7.5 mg/15 ml) at 37°C for 16 hours, the DNA is stored at 4°C. In this manner, 1 ml of solution containing 0.6 mg of DNA is obtained.

70 µg of this DNA are partially digested with enzyme MboI (10 minutes; 40 units) and then applied to a linear sucrose gradient (20% - 40%). After centrifugation (SW28 rotor, 16 hours at 25,000 rpm, 20°C), 500 µl fractions are collected. Those fractions containing DNA between 10 and 20 kb in size (fraction 23-26) are combined, dialysed against TE buffer, then precipitated with ethanol. In this manner, 5 µg of DNA are obtained.

In order to provide a cloning vector for the DNA described above, the DNA of bacteriophage lambda EMBL 301 (19) is digested with restriction enzyme BamHI. After extraction

with phenol-chloroform and precipitation with ethanol, the DNA is resuspended in TE buffer.

The genomic DNA from the CaSki cells and the lambda bacteriophage DNA are then ligated in accordance with a conventional protocol (1  $\mu$ g of vector, 2  $\mu$ g of genomic DNA). After packaging the DNA using a kit marketed by Amersham, a total of  $0.75 \times 10^6$  independent clones is obtained. These bacteriophages are spread with *E. coli* Q358 bacteria onto eighteen 14 cm diameter dishes on LBM medium in order to be screened with the synthetic oligonucleotides inferred from the sequence of the HPV-16 virus [(EMBL) PA16].

Screening of the recombinant bacteriophages is performed in the customary manner for the person skilled in the art. The oligonucleotides 1817 (sequence 5' CATGCATGGAGATACACCTACATTG 3'; reading frame E7) and 1818 (sequence 5' GTGGATAACAGCAGCCTCTGCGTTT 3'; reading frame E5), radioactively labelled with  $^{32}$ P are mixed and hybridised with phage DNA (transferred onto nitrocellulose filters) for 16 hours at 55°C in 6  $\times$  concentrated SSC buffer. The filters are then washed under conditions of the same stringency, 10 positive signals (1-10) are obtained. The area of the Petri dishes corresponding to these signals is taken up in 1 ml of LBM buffer. These suspensions are again spread onto two sets of 10 cm  $\varnothing$  Petri dishes (2-10  $\mu$ l of suspension per dish) and a second screening is performed with oligonucleotides 1817 and 1818 separately. Since the signals corresponding to the phages obtained from the first isolations 4 and 5 are the most intense, these two clones are selected for the continuation of the experiments.

Minicultures of *E. coli* Q358 bacteria infected with the bacteriophage isolated from sub-clones 4-1, 4-2, 5-1 and 5-2 are made. The DNA from these clones is purified and digested with restriction enzyme PstI in order to analyse the selected recombinants.

In order to limit sequencing work and since the DNA sought are those corresponding to reading frames E5, E6 and E7, only those regions of the PstI restriction fragments

corresponding to proteins E6 and E7 (M13 E7/E6) and E5 (M13 E5) are sequenced with synthetic oligonucleotides with the sequence

5' ATCTAACATATATTC 3' and 5' GTTGTTCCATACAAA 3' for M13 E7/E6

and

5' GTCTGCCTATTAATAC 3' for M13 E5.

Figure 4 represents the structure of bacteriophage M13 E7/E6 and figure 5 represents the structure of bacteriophage M13 E5. The sequence obtained for E7 reveals complete homology with the sequence contained in the PPH16 databases. Two mutations are observed for E6: G instead of A in position +46 and G instead of T in position 264 in relation to the start ATG of E6. The sequence shown in figure 6 is obtained for E5, a few bases of which sequence differ from the sequence contained in the database.

Example 4: Cloning of DNA fragments bearing E6, E7 and E5 in the transfer vector.

Before incorporating the DNA coding for these three reading frames into the recombinant vaccinia viruses, it is necessary to modify them in order to obtain unique restriction sites upstream and downstream from the genes and to improve the sequences around the start ATG in order to obtain a good translation and a high level of synthesised protein.

a) Reading frame E6

A SalI restriction site and a SphI site are respectively created upstream and downstream from the sequence coding for E6 with two synthetic oligonucleotides, using an oligonucleotide-directed localised mutagenesis technique

(Amersham kit). These two localised mutations are performed simultaneously using the following oligonucleotides:

5' GTTAGTATAAAAGTCGACCCACCATGCACCAAAGAG 3'  
5' CATGCATGCAGATACACC 3'

An A nucleotide is introduced in position -3 in relation to the ATG instead of a T nucleotide at the same time as the SalI site.

The bacteriophages obtained are analysed by digestion with restriction enzymes and by sequencing. A clone is selected for the continuation and designated M13TG1181. The SalI-SphI restriction fragment is then cloned into the pTG186poly vector (11) open at the SalI and SphI sites (pTG2198). This vector allows the transfer of the DNA into the vaccinia virus genome.

b) Reading frame E7

A PstI restriction site and an A nucleotide in position -3 are introduced upstream from the sequence coding for protein E7 by localised mutagenesis using the following oligonucleotide:

5' GTAGAGAAACCCTGCAGCCACCATGCATGGAG 3'

Several clones were obtained. One of them, containing a 350 bp PstI restriction fragment, is sequenced in the zone corresponding to the mutation. The sequence does indeed correspond to the expected mutation (M13TG1182). The PstI restriction fragment is then inserted into plasmid pTG186poly open at site PstI (pTG2199).

c) Reading frame E5

A PstI restriction site is inserted upstream from the start ATG of reading frame E5 (position 3851 in PPH16) by localised mutagenesis using the following oligonucleotide:

5' GTCTACTGGATTTACTGCAGTATGACAAATCTTGAT 3'

An EcoRI restriction site is introduced downstream from the stop codon using the following oligonucleotide:

5' GTATATGTACATAATGAATTCTTACATATAATTGTTG 3'

These two mutations are introduced simultaneously (Amersham kit). The clone selected for the continuation is designated M13TG 3151. The PstI-EcoRI restriction fragment is then inserted into plasmid pTG186poly open at the sites PstI and EcoRI (pTG3180).

Example 5: Construction of recombinant vaccinia viruses.

Plasmids pTG2198, 2199 and 3180 are used to transfer reading frames E6, E7 and E5 into a vaccinia virus (Copenhagen strain) as previously described. The recombinant viruses, in which the coding sequences are under the control of the vaccinia 7.5 k promoter, are named VVhE6, VVhE7 and VVhE5.

BHK-21 cells on MEM.BME medium supplemented with 10% foetal calf serum are infected with one of the three recombinant viruses with a plurality of approximately 20 pfu per cell.

After incubation in accordance with the previously described protocol, the cells are recovered, lysed and fractionated in order to determine the subcellular location of the HPV-16 proteins.

Expression of the E6 and E7 genes is verified by immunoprecipitation of the proteins synthesised (labelled with  $^{35}\text{S}$  cysteine).

The polyclonal antibodies directed against the bacterial fusion protein corresponding to reading frames E6 and E7 are obtained by the conventional method known to the person skilled in the art and described in references (20) and (21).

The cells infected with VVhE6 produce an 18 kDa cytoplasmic protein which is specifically recognised by anti-E6 antiserum (figure 7, band 1, band 2 corresponds to immunoprecipitation with a non-immune serum. The cells infected with VVhE7 also produce a 19-20 kDa cytoplasmic protein specifically recognised by anti-E7 antiserum (figure 7, band 3, band 4 corresponds to immunoprecipitation with non-immune serum).

Since no anti-serum is available for E5, incorporation of the E5 expression block into the vaccinia virus was verified by DNA analysis using the Southern technique.

Example 6:        Effect of vaccination with VVhE6 and VVhE7 on the development of tumours induced by HPV-16.

Groups of 4 week old female (Fischer) rats are vaccinated by intradermal injection of  $5 \cdot 10^7$  pfu (in 100  $\mu\text{l}$ ) of the various recombinant viruses. A booster injection with the same dose is administered after 12 days.

Between the 16th and 17th day, they are tested with a line obtained by co-transfection into primary rat cells: (line RE01)

- 1) of a plasmid including the HPV-16 genome under the control of the Moloney retrovirus LTR (22);

- 2) of a plasmid coding for a resistance gene to G418 and for EJ-RAS, which transforms only cells of established lines or expressing an immortalising gene.

$2 \times 10^4$  cells are injected subcutaneously in a 200  $\mu$ l volume of MEM.BME buffer without serum.

The following observations are made on the basis of figures 8 and 9:

- unvaccinated control animals develop detectable tumours 10 days after the inoculation of transformed cells (see dashed lines).
- animals vaccinated with VVhE6 do not develop tumours in 2 cases out of 7 (> 100 days). When tumours appear, in three cases their development is very significantly slowed and for two animals the appearance of tumours is delayed (24 and 34 days instead of 10 days). Finally, in two cases, tumour development is identical to the development found in the animals in the control batch.
- animals vaccinated with VVhE7 do not develop tumours in 3 cases out of 7 (> 100 days). In the four other cases, tumours appear from the 10th days, but their development is significantly delayed.

In order to test the protective effects of these vaccinations, those animals in the two batches which did not develop tumours are taken and tested 80 days after the first inoculation test with 10 times the initial dose, i.e.  $2 \times 10^5$  cells. Under such inoculation conditions, control animals develop tumours after 4 days.

- animals which had been vaccinated with VVhE6 develop tumours in an identical manner to the control animals.
- in contrast, the animals previously vaccinated with VVhE7 do not develop tumours even under these conditions (> 100 days).

These results and the preceding ones emphasise the important role played by E7 antigens in protection against the development of tumours induced by HPV-16.

In order to verify the above results, two further series of experiments are performed with other cell lines. Groups of 4 week old female rats are vaccinated by intradermal injection with recombinant viruses as previously described. The rats vaccinated with the recombinant viruses are tested with  $2 \cdot 10^4$  or  $10^5$  primary rat cells (line RE31) prepared according to the previously described protocol. The number of animals rejecting tumours or in which a delay in the appearance of tumours is observed is given in the first part of table II (first test). In this table, VV0 corresponds to a control recombinant vaccinia virus which does not express a HPV protein. Then, some rats which had rejected the tumours a first time are re-tested but using a higher concentration of primary cells ( $2 \cdot 10^5$  instead of  $2 \cdot 10^4$  or  $10^5$ ). The results obtained are shown in the second part of table II (second test).

TABLE II

| Recombinant virus                                                                        | Rejection | Delay in appearance of tumour | Total |
|------------------------------------------------------------------------------------------|-----------|-------------------------------|-------|
| <u>First test by injection of <math>2 \times 10^4</math> cells of line RE31 per rat</u>  |           |                               |       |
| VVhE6                                                                                    | 3/18      | 5/18                          | 8/18  |
| VVhE7                                                                                    | 3/17      | 5/17                          | 9/17  |
| VV0                                                                                      | 0/17      | 0/17                          | 0/17  |
| <u>First test by injection of <math>10^5</math> cells of line RE31 per rat</u>           |           |                               |       |
| VVhE6                                                                                    | 7/20      | 1/20                          | 8/20  |
| VVhE7                                                                                    | 5/20      | 0/20                          | 5/20  |
| VV0                                                                                      | 1/20      | 0/20                          | 1/20  |
| <u>Second test by injection of <math>2 \times 10^5</math> cells of line RE31 per rat</u> |           |                               |       |
| VVhE6                                                                                    | 2/4       | 0/4                           | 2/4   |
| VVhE7                                                                                    | 3/4       | 1/4                           | 4/4   |
| VV0                                                                                      | 0/5       | 0/5                           | 0/5   |

In another series of experiments, primary rat cell lines, used to test the vaccinated rats, are transfected by:

- 1) a plasmid including the HPV-16 genome under the control of its own promoter,
- 2) a plasmid coding for a resistance gene to G418 and for EJ-RAS which transforms only cells of established lines or expressing an immortalising gene.

In this way, line RE604 is obtained. Table III presents the results obtained under these experimental conditions.

TABLE III

Test by injection of  $2 \times 10^4$  cells of line RE604 per rat

| Recombinant virus | Rejection | Delay in appearance of tumour | Total |
|-------------------|-----------|-------------------------------|-------|
| VVhE6             | 0/30      | 6/30                          | 6/30  |
| VVhE7             | 7/40      | 10/40                         | 17/40 |
| VVhE6/VVhE7       | 0/10      | 2/10                          | 2/10  |
| VV0               | 0/30      | 0/30                          | 0/30  |

The results of these experiments emphasise the important roles played by antigens E7 and E6 in protection against tumours induced by HPV-16.

In all the cell lines, expression of protein E7 is verified by immunoprecipitation. The protective action exercised by antigen E7 is therefore not dependent upon the line in question.

The following strains were deposited on 24 February 1989 with the *Collection Nationale de Cultures de Microorganismes* of the *Institut Pasteur* (Paris):

- *E. coli* pTG2198 under n° I-837.
- *E. coli* pTG2199 under n° I-838.
- *E. coli* pTG3180 under n° I-839.

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**CLAIMS (except for Spain)**

- 1) Pharmaceutical preparation intended for the treatment or prevention of a papillomavirus infection or tumour, which preparation comprises a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus or for an essential region of the said protein; the said DNA fragment being placed under the control of the factors necessary for its expression in mammalian cells.
- 2) Pharmaceutical preparation according to claim 1 comprising a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus; the said DNA fragment being placed under the control of the factors necessary for its expression in mammalian cells.
- 3) Pharmaceutical preparation according to claim 2 comprising a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a type HPV-16 papillomavirus.
- 4) Pharmaceutical preparation according to one of claims 2 or 3 comprising a recombinant vaccinia virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus.



- 5) Pharmaceutical preparation according to one of claims 2, 3 or 4 comprising a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>5</sub> originating from a papillomavirus.
- 6) Pharmaceutical preparation according to one of claims 2, 3 or 4 comprising a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>6</sub> originating from a papillomavirus.
- 7) Pharmaceutical preparation according to one of claims 2, 3 or 4 comprising a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>7</sub> originating from a papillomavirus.
- 8) Pharmaceutical preparation according to one of claims 1 to 7, characterised in that position -3 of the translation initiation region comprises an A or a G.
- 9) Pharmaceutical preparation according to one of claims 1 to 8, characterised in that the pox virus is living or killed.
- 10) Pharmaceutical preparation according to one of claims 1 to 9, characterised in that it comprises an acceptable pharmaceutical carrier allowing its administration by injection into man or animals.



**CLAIMS FOR SPAIN**

- 1) Process for the production of a pharmaceutical preparation intended for the treatment or prevention of a papillomavirus infection or tumour, which preparation comprises the formulation of a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus or for an essential region of the said protein; the said DNA fragment being placed under the control of the factors necessary for its expression in mammalian cells, with an acceptable pharmaceutical carrier.
- 2) Process according to claim 1 in which the pox virus is a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus; the said DNA fragment being placed under the control of the factors necessary for its expression in mammalian cells.
- 3) Process according to claim 2 in which the pox virus is a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a type HPV-16 papillomavirus.
- 4) Process according to one of claims 2 or 3 in which the pox virus is a recombinant vaccinia virus, into the genome of which is inserted a fragment of heterologous DNA coding for a protein selected from the proteins E<sub>5</sub>, E<sub>6</sub> and E<sub>7</sub> originating from a papillomavirus.



- 5) Process according to one of claims 2, 3 or 4 in which the pox virus is a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>5</sub> originating from a papillomavirus.
- 6) Process according to one of claims 2, 3 or 4 in which the pox virus is a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>6</sub> originating from a papillomavirus.
- 7) Process according to one of claims 2, 3 or 4 in which the pox virus is a recombinant pox virus, into the genome of which is inserted a fragment of heterologous DNA coding for protein E<sub>7</sub> originating from a papillomavirus.
- 8) Process according to one of claims 1 to 7, characterised in that position -3 of the translation initiation region comprises an A or a G.
- 9) Process according to one of claims 1 to 8, characterised in that the pox virus is living or killed.
- 10) Process according to one of claims 1 to 9, characterised in that the acceptable pharmaceutical carrier allows its administration by injection into man or animals.