(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number
WO 02/29074 A2

(51) International Patent Classification^7: **C12N 15/86**

(21) International Application Number: PCT/NL01/00740

(22) International Filing Date: 8 October 2001 (08.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00203472.6 6 October 2000 (06.10.2000) EP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

(54) Title: GENETIC IMMUNISATION AGAINST CERVICAL CARCINOMA

(57) Abstract: The invention provides a method for the treatment or prevention of cervical cancer comprising providing an individual with a medicament comprising an alphavirus vector system(s) and/or a cell(s) comprising nucleic acid derived from human papilloma virus (HPV) for the preparation of a medicament wherein said medicament is a vaccine for the treatment of cervical cancer.

Title: Genetic immunisation against cervical carcinoma

The invention relates to the field cervical cancer caused by human papillomaviruses. Papillomaviruses are small naked DNA tumour viruses (7.9Kb, double stranded), which are highly species specific. Over 90 individual human papilloma viruse types (HPV) have been described. Genital HPV infection in young sexually active women is common and most individuals either clear the infection, or if lesions develop these regress. Only a subset of infected individuals have lesions which progress to a high grade intraepithelial neoplasia and only a fraction of these progress further to invasive carcinoma. Carcinoma of the cervix in woman develops through a pre-cancerous intermediate stage to an invasive carcinoma which leads frequently to death. Infection of genital epithelial cells with human papillomavirus (HPV) types 16 and 18 is closely associated with the development of cervical carcinoma. The HPV genome encodes 7 early (E) nonstructural regulatory proteins, and two late (L) structural proteins. Integration of the viral DNA in the genome of the host cell, which is considered an essential step in HPV16 or HPV18 induced development of cervical carcinoma, results in a loss of E1 or E2 mediated transcriptional control. As a consequence the transformed cells over-express the E6 and E7 proteins, initiating the malignant transformation process [Pei (1996) Carcinogenesis 1996; 17: 1395-1401].

Specific cell-mediated immunity is believed to play an essential role in the control of HPV infections and cervical carcinoma. This assumption is based on observations showing (i) that HPV-induced lesions regress spontaneously in the majority of individuals, and (ii) that immunodeficient patients develop significantly more HPV related proliferative lesions in skin and anogenital tissue than immunocompetent individuals. In several animal models it has been demonstrated that the HPV E6 and E7 proteins, constitutively expressed in HPV transformed cells, can act as targets for CTL mediated tumor cell killing and stimulation of tumor specific CTL activity. Induction of an antigen-specific CTL response requires intracellular processing of the target antigen and presentation of antigenic peptides by MHC class I molecules.

In the last few years a number of peptide/protein-based or genetic immunization strategies have been described for the induction of HPV specific CTL activity. Major drawbacks associated with a peptide-based approach include the problem of MHC-polymorphism and the risk of inducing T cell tolerance rather than T cell activation. Due to the induction of specific T cell tolerance, vaccination with a tumor-specific peptide has been shown to result in an enhanced outgrowth of the tumor. Immunization with larger proteins would overcome these problems, but this requires efficient antigen delivery systems and/or safe adjuvants for efficient immune priming. The induction of HPV specific CTL responses in mice upon immunization with recombinant vaccinia virus expressing HPV E6 or E7 unexpectedly produced lower titres compared with the parental strain which seriously reduces the effectiveness for inducing HPV specific CTL responses. Other drawbacks associated with the use of the vaccinia virus based vector system are immune responses against viral proteins in pre-immune patients or more seriously integration of recombinant genes into the host cell genome (retrovirus). Especially, when the recombinant virus encodes oncoproteins such as HPV E6 or E7, the risk of integration into the host cell genome is a point of major concern as infected cells can indeed survive and become tumorigenic (immortalised).

The invention provides an alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV). Alphaviruses consist of a nucleocapsid with one copy of a single-stranded RNA molecule surrounded by an envelope containing spike proteins. Alphavirus RNA has a positive polarity enabling the genomic RNA to initiate an infection when introduced into the cytoplasm of a cell. In addition, the RNA is self-replicating, since it encodes its own replicase, and replication results in high-level expression of the viral proteins in host cells. Nucleic acid sequence or nucleic acid molecule as used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represents the sense or antisense strand. The definition ‘antisense’ RNA is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or majority of bases) in the antisense strand (read in the 5' to 3' sense) is capable of pairing with the corresponding base (G with C, A with U), in the mRNA sequence read in the 5' to 3' sense. The definition 'sense' RNA is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. A preferred embodiment is that the nucleic acids are derived from a human papilloma virus (HPV) type 16 and/or type 18. The invention provides a nucleic acid which can be a gene or a functional part of a gene (wherein a gene is a nucleic acid which can be expressed) or a precursor of a gene or a transcribed gene on any nucleic acid level (DNA and/or RNA: double or single stranded) and/or gene product derived thereof that can overcome cell cycle suppression by inactivating major tumour supressor proteins, P53 and pRB (retinoblastoma) gene products, respectively leading to loss of normal cellular differentiation and the development of a carcinoma. A gene product herein refers to mRNA and the polypeptide chain translated from an mRNA molecule, which in turn is transcribed from a gene; if the RNA transcript is not translated (e.g rRNA, tRNA) the RNA molecule represents the gene product. The gene product herein refers to any proteinaceous substance. A proteinaceous substance refers to any molecule comprising peptide or protein. In a preferred embodiment the invention provided an alphavirus vector wherein said nucleic acid is derived from the human papilloma virus (HPV) type 16 and/or type 18, E6 and E7 oncogenes or functional fragments or derivatives thereof which are involved in transformation. 'Functional fragment or derivatives thereof' herein means that the subject signature sequence can vary from the reference sequence by one or more substitutions, deletions, or additions, the net effect of which will not result in a functional dissimilarity between the two sequences. It is known by those skilled in the art that as a result of degeneracy of the genetic code, a multitude of gene sequences, some bearing minimal homology to the nucleotide sequences of any known and any naturally occurring genes may be produced. The invention contemplates each and every possible variation of the nucleic acid that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code. All such variations are to be considered as being specifically disclosed. Theoretically a minimal fragment length would be 9 AA since this is the length of the average CTL-epitope. Vaccination with peptides of this length indeed results in a CTL response.

However, preferred is to use constructs that encode longer peptides/proteins such that the preparations are not limited by HLA-restriction.

The invention provides an alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV), wherein said nucleic acid encodes at least one antigenic polypeptide fragment of said HPV. An ‘antigenic polypeptide fragment’ as used herein refers to at least one gene product or fragment thereof derived from said HPV nucleic acid, wherein said gene product comprises a proteinaceous substance(s) wherein said proteinaceous substance is an antigen (foreign invader which is usually a protein or protein attached moiety) capable of initiating an immune response. An ‘immune response’ herein refers to the physiological response(s) stemming from activation of the immune system by antigens. A preferred embodiment is where said immune response involves the production HPV-specific cytotoxic T lymphocyte (CTL).

The invention provides an alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV), wherein said nucleic acid encodes at least one antigenic polypeptide fragment of said HPV, wherein said antigenic polypeptide fragment is derived from protein E6 and/or protein E7 of HPV. E6 and E7 are viral oncogenes. More preferably the gene product comprises an antigenic polypeptide fragment derived from E6 protein and/or E7 protein of HPV. E6 and E7 protein refer to oncoproteins derived from E6 and E7 HPV genes, respectively and are viral associated products expressed in cervical cancer which can immortalize target cells. The expression of E6 and E7 genes is selectively maintained in pre-malignant and malignant cervical lesions and their gene products contribute to the transformation process and are necessary to maintain the transformed state.

The invention also provides an alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV) wherein said nucleic acid encodes at least one antigenic polypeptide fragment of said HPV, wherein said antigenic polypeptide fragment comprises one antigenic polypeptide fragment of E6 and one antigenic polypeptide fragment of E7. Antigenic polypeptide fragment of HPV herein refers to tumour antigens E6 and E7 that can bind to the cellular tumour suppresser products pRB and P53. Preferably, nucleic acid encoding E6 and E7 or fragments thereof are fused in frame.

The invention further provides an alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV) wherein said nucleic acid encodes at least one antigenic polypeptide fragment of said HPV wherein said antigenic polypeptide fragment is at least partially been deprived of the capacity to bind to pRb and/or P53 protein. The retinoblastoma (pRb) gene and the p53 gene encodes tumor suppresser gene products that control cell proliferation. Loss of activity of both genes causes unrestrained cell growth. E7 is a cytoplasmic serine phosphoprotein, and has been shown to be a transcriptional transactivator and transforming protein. E7 binds Rb protein and then presumably moves to nucleus. E6 binds to a cellular protein called E6-Ap. This complex then binds to p53. E6-Ap is a ubiquitin ligase. Cellular enzymes load it with activated ubiquitin molecules which are transferred to p53. Ubiquitin loading of p53 targets it for degradation by proteasome mediated proteolysis. The forced entry into S-phase in conjunction with genomic instability, resulting from p53 degradation may lead to malignancy. E6/7 proteins from 'low risk' HPV strains appear not to associate with Rb and p53. Expression of E6/7 antisense constructs reduces cell growth indicating that E6/7 may not only participate in initiation but also maintain the proliferative and malignant phenotype.To overcome the risk of an infected cell virus expressing E6 and E7 surviving to become tumorigenic (immortalized), a risk associated with other vector systems (i.e. vaccinia virus) used in the prior art, the invention provides for a recombinant virus expressing E6 and E7 oncogenes devoid of the capacity to suppress the retinoblastoma (pRb) and p53 gene products. Thus cells infected with the virus are maintained in a non-tumorigenic (or oncogenic) state. Methods to reduce DNA-protein interaction and protein-protein interaction are known. These include but are not limited to protein engineering. Protein engineering herein refers to any biochemical technique by which novel protein molecules are produced. These techniques can include the *de-novo* synthesis of protein through assembly of functional units from different natural proteins and through introduction of small changes such insertions, deletions and substitutions in the nucleotide or protein sequence. A ‘deletion’ is defined as a change in either nucleotide or protein sequence in which one or more nucleotides or amino acid residues, respectively, are absent. An 'insertion' or 'addition' is that change in nucleotide or protein sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring polypeptide(s). A 'substitution' results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The invention further provide an alphavirus vector system comprising an translational enhancer element. To overcome lower expression or improve on expression of heterologous proteins than that obtained for structural viral proteins during a wild-type SFV infection, the invention provides for an alphavirus vector system comprising a translational enhancer element, wherein said translational enhancer element preferably comprises a viral capsid gene segment, more preferably from an alphavirus (e.g.Semliki Forest virus (SFV)). A translational enhancer element herein is defined as a functional sequence segment from for example prokaroyte, eukaryote, viral origin etc. that can increase the utilization of promoters, and can function in either orientation and in any location (i.e upstream or downstream) relative to the promoter. Provided of course is use of enhancer elements in general that can enhance heterologous protein synthesis of an alphavirus vector system. The effect is of such enhancers is likely mediated by the mRNA sequence acting in cis during initiation of protein translation. Further, the original location of the initiator AUG is important for such enhancer effect.

In one embodiment, he invention further provides an alphavirus vector system wherein the alphavirus capsid and spike proteins are expressed from at least one nucleic acid molecule. The alphavirus capsid and spike proteins are viral structural proteins. The alphavirus system the subject of the invention more preferably a Semiliki Forest Virus based system has enhanced biosafety over currently used viral based systems. By splitting the capsid region which contains a translational enhancer and spike protein region into two independant RNA molecules and by co-transfecting a cell with these two independant RNA molecules and the SFV vector replicon, RNA recombination is negligible and so is the production of replication efficient viruses. In addition by abolishing autoprotease activity of the capsid protein increase the safety of the system means that the production of replication efficient viruses is further prevented. In another embodiment, he invention further provide an alphavirus vector system wherein the alphavirus capsid and spike proteins are expressed from at least two independent nucleic acid molecules. Such vector systems as referred to above are commonly called helper-2-system and 2-helper or split helper system.

The invention further provide an alphavirus vector system wherein said alphavirus comprises Semliki Forest Virus (SFV). More preferably the alphavirus vector system is the Semliki Forest virus (SFV) expression system. SFV belongs to the genus Alphavirus of the family of the Togaviridae. A full-length cDNA copy of the SFV viral genome has been cloned in a bacterial plasmid including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed in vitro. These RNA transcripts are fully infectious (i.e. introduction into cells suffices to initiate replication and a full infection cycle, resulting in virus formation). The Semliki Forest virus (SFV) expression system allows for efficient expression of foreign coding sequences as part of the SFV RNA replicon. The SFV viral system is especially suited to safely induce cellular immune responses against oncoproteins such as HPV 16/18 E6 and E7. Firstly, SFV is an RNA virus replicating in the cell cytosol so there is no risk of integration of the E6 and E7 genes in the cellular genome. Moreover, SFV infection is cytolytic by apoptosis, therefore no genetic information of E6 and E7 will likely persist for more than one week after injection. In addition, no other vector proteins are produced, besides small amounts of viral replicase. Immune responses against the SFV vector itself did not inhibit boost responses by subsequent immunizations with the same vector, a problem associated with other viral vector systems. Furthermore, as further explained in the detailed description herein, a full-length cDNA copy of the SFV viral genome has been cloned in a bacterial plasmid including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed in vitro. These RNA transcripts are fully infectious (i.e. introduction into cells suffices to initiate replication and a full infection cycle, resulting in virus formation). The Semliki Forest virus (SFV) expression system allows for efficient expression of foreign coding sequences as part of the SFV RNA replicon.

The invention further provides an alphavirus vector system wherein said HPV comprises HPV16 and/or HPV18. More preferably the alphavirus vector system comprises a Human papillomavirus (HPV) based vector system, and even more preferable a HPV vector system based on HPV types 16 and 18 which are more closely associated with the development of cervical carcinoma. Included are viral variants of all preferred viral vector systems of the present invention.

The invention further provides an alphavirus vector system or other viral vector systems according to the present invention wherein said nucleic acid further encodes a cytokine gene or functional fragment thereof. Cytokines are primarily involved in signaling between cells of the immune system. It is herein provided to use Granulocyte-Macrophage Colony-Stimulating-Factor (GM-CSF) and/or Interleukin 12 (IL-12), however, one could also consider IL-2, IL-6, IL-18, and others too. It is herein also provided to use separate vector particles, for example SFV particles encoding cytokines and SFV particles encoding eE6,7. The particles not necessarily act at the same time/site, by making separate particles the preparations can be given separately (in time/route/dosage). The definition 'functional fragment' herein means a fragment (i.e reference sequence) derived from the subject sequence (e.g a cytokine gene) which may vary from the subject sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and the subject sequence.

The invention further provides a cell comprising an alphavirus vector system(s) or other viral vector systems according to the invention. Methods to infect a target cell with a recombinant virus of the present invention are known. The invention further provides the use of an alphavirus vector system(s) according to the present invention and/or a cell infected with an alphavirus vector system(s) according to the present invention for the preparation of a medicament. A preferred embodiment is a medicament which is a pharmaceutical. Compositions for the preparation of a medicament, and methods to deliver said medicament comprising a recombinant alphavirus and/or a cell comprising said recombinant alphavirus the subject of the invention are provided herein as well. The invention further provides the use of an alphavirus vector system(s) according to the present invention and/or a cell infected with an alphavirus vector system(s) according to the present invention for the preparation of a medicament for the treatment of cervical cancer.

The invention further provides the use of an alphavirus vector system(s) according to the present invention and/or a cell(s) infected with an alphavirus vector system(s) or other viral vector systems according to the present invention for the preparation of a medicament wherein said medicament is a vaccine. Vaccine compositions and methods to administer a vaccine to a patient are well known in the art. In a preferred embodiment the invention provides a biosafe method to vaccinate against cervical cancer comprising providing an alphaviral vector system with a broad host range comprising nucleic acid encoding tumour antigens devoid of capacity to bind to the cellular tumour supressor products pRB and P53 and capable of inducing a HPV-specific cytotoxic T lymphocyte (CTL) response against HPV-transformed tumor cells expressing tumour antigens. CTLs can destroy cells expressing foreign antigens through recognition of foreign peptides generated within the cell and transported to the cell surface and presented by histocompatibility complex (MHC) class I antigens. They are thus potentially powerful agents of tumour cell destruction.

The invention further provides the use of an alphavirus vector system(s) according to the present invention and/or a cell(s) infected with an alphavirus vector system(s) or other viral vector systems according to the present invention for the preparation of a medicament wherein said medicament is a vaccine for the treatment of cervical cancer. In a preferred embodiment such recombinant alphaviruses comprise at least nucleic acid derived from HPV, more preferable HPV E6 and/or HPV E7 viral oncogenes, derived from HPV type 16 and/or 18, in order to immunize the body against preferably HPV type 16 and/or 18 for the treatment and prevention of cervical cancer.

The invention further provides a method for the treatment or prevention of cervical cancer comprising providing an individual with a medicament comprising an alphavirus vector system(s) according to the present invention and/or a cell(s) infected with an alphavirus vector system(s) according to the present invention for the preparation of a medicament wherein said medicament is a vaccine for the treatment of cervical cancer.

Detailed description

Genetic immunization against cervical carcinoma

From molecular, clinical and epidemiological studies it is evident that the high-risk human papilloma viruses HPV16 and HPV18 are linked to the development of precursor lesions of cervical cancer and invasive cervical carcinoma. The HPV genome encodes 7 early (E) nonstructural regulatory proteins, and two late (L) structural proteins. Integration of the viral DNA in the genome of the host cell, which is an essential step in HPV16- or HPV18-induced development of cervical carcinoma, results in a loss of E1- or E2-mediated transcriptional control. As a consequence the transformed cells overexpress the E6 and E7 proteins, initiating the malignant transformation process.

Specific cell-mediated immunity is believed to play an essential role in the control of HPV infections and cervical carcinoma. This assumption is based on observations showing (i) that HPV-induced lesions regress spontaneously in the majority of individuals, and (ii) that immunodeficient patients develop significantly more HPV-related proliferative lesions in skin and anogenital tissue than immunocompetent individuals. In several animal models it has been demonstrated that the HPV E6 and E7 proteins, constitutively expressed in HPV-transformed cells, can act as targets for CTL-mediated tumor cell killing and stimulation of tumor-specific CTL activity.

Induction of an antigen-specific CTL response requires intracellular processing of the target antigen and presentation of antigenic peptides by MHC class I molecules. This can be achieved efficiently with recombinant viral vectors. Heino P. et al.: "Human papillomavirus type 16 capsid proteins produced from recombinant Semliki Forest virus assemble into virus-like particles" describes a method for the production of HPV16 virus-like particles (VLP's). These VLP's consist of the two HPV16 structural proteins, L1 and L2. Upon expression of L1 and L2 in producer cell cultures, the cells generate VLP's. In this paper, the Semliki Forest virus (SFV) vector system is used to express L1 and L2 in a producer cell with the purpose of generating HPV16 VLP's. The paper does not relate to the development of an alphavirus vector for genetic immunization against cervical carcinoma. Accordingly, it does not relate to expression of the HPV16 tumor antigens E6 and E7. The paper is only indirectly related, in that HPV16 VLP's consisting of L1 and L2 could be used as a vaccine for induction of an antibody response against the virus HPV16. However, such a response would not be directed against HPV16-induced tumors since such tumors only express the viral tumor antigens E6 and E7.

Boursnell M.E.G. et al.: "Construction and characterisation of a recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer" describes the construction and characterization of a recombinant vaccinia virus vector (TA-HPV) expressing the tumor antigens E6 and E7 from HPV16 or HPV18. It is demonstrated that the recombinant virus, upon intraperitoneal administration to mice, has the capacity to prime a cytotoxic T lymphocyte (CTL) response against cells infected with the same virus vector or sensitized with a synthetic E7 peptide epitope. The alphavirus vector disclosed in the present invention has several distinct and important advantages over the TA-HPV vector. First, the alphavirus vector is replication-incompetent, whereas the vaccinia virus vector, which is based on attenuated live poxvirus, is replication-proficient. Replication-proficiency represents a major safety problem, the TA-HPV vectors carrying potentially oncogenic sequences derived from HPV16 or HPV18.

Second, vaccinia virus is a DNA virus, whereas alphaviruses are RNA viruses, which replicate and translate their RNA in the cytosol of cells without involvement of any nuclear processing. Therefore, genes encoded by an alphavirus vector can not become integrated in the genome of the cell, whereas in the case of DNA virus vectors this possibility can never be excluded. The exclusive cytosolic RNA processing of the genome of alphavirus vectors represents an important safety feature of these systems.

Third, the alphavirus vector disclosed in the present invention contains a translational enhancer in front of the antigenic sequence derived from HPV. This translational enhancer ensures high levels of antigen expression in target cells. Thus, the disclosed alphavirus vector is considerably more efficient than the TA-HPV vector, inducing higher levels of CTL activity with smaller dosages of virus.

EP 0 711 829 Arelates to one specific subcategory of alphavirus vectors, i.e. Sindbis virus vectors. Our present invention discloses another alphavirus vector, (derived from Semliki Forest virus), encoding an antigenic sequence of a HPV. While it is indicated that foreign antigenic sequences may be included in the Sindbis-derived vector (many possibilities are mentioned, including sequences derived from an HPV), none of the examples involves expression of HPV-derived E6 and/or E7 antigenic sequences, but relate to expression of antisense sequences to E6 and/or E7 instead in an entirely different approach aimed at inhibition of E6 and/or E7 expression rather than stimulation of an immune response against E6 and/or E7., let alone induction of a cytotoxic T lymphocyte response using the Sindbis-derived vector, as is provided in the present invention. Furthermore, the Sindbis-derived vector does not comprise a translational enhancer sequence.

WO 99 28487 A (Crown in the right of the Queen; Khromykh; Varnavs) "Flavivirus expression and delivery systems" discusses a flavivirus expression system. It does not involve alphavirus expression vectors. Flaviviruses comprise a different family of positive-strand RNA viruses, i.e. the *Flaviridae.* Alphaviruses belong to the family *Togaviridae.*

Borysiewicz L.K. et al.: "A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer." describes the outcome of a first human clinical trial with a live recombinant vaccinia virus vector expressing the E6 ane E7 proteins of HPV16 and HPV 18 (TA-HPV). The construction and characterization of the TA-HPV vector is described in Boursnell et al. Berglund P. et al.: "Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice" describes an immunization study in mice, utilizing the recombinant SFV vector system encoding the influenza virus nucleoprotein or *E. coli LacZ.* It does not relate to SFV encoding HPV-derived antigens.

In the present study, our approach is to use the Semliki Forest virus (SFV) expression system. SFV belongs to the genus *Alphavirus* of the family of the *Togaviridae.*[13] Alphaviruses consist of a nucleocapsid with one copy of a single-stranded RNA molecule surrounded by an envelope containing spike proteins. Alphavirus RNA has a positive polarity enabling the genomic RNA to initiate an infection when introduced into the cytoplasm of a cell. In addition, the RNA is self-replicating, since it encodes its own replicase, and replication results in high-level expression of the viral proteins in host cells. A full-length cDNA copy of the viral genome has been cloned in a bacterial plasmid including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed *in vitro*. These RNA transcripts are fully infectious, i.e. introduction into cells suffices to initiate replication and a full infection cycle, resulting in virus formation.[14] Liljeström and coworkers[14-16] developed a vector system that allows for efficient expression of foreign coding sequences as part of the SFV RNA replicon. A high biosafety level is obtained by separating the replicase and structural genes of the viral genome. Thus, recombinant virus particles can be produced that infect cells only once. In addition, the SFV helper (containing the structural genes) was mutated in the gene encoding one of the spike proteins.[15] In effect, such virus particles cannot infect cells unless they are activated with exogenous protease.

Detailed description

Example 1

Here we describe the construction of recombinant SFV encoding HPV16 E6 and E7 and the cellular immune response in mice induced by these recombinant SFV-E6E7 particles. Infection of genital epithelial cells with human papillomavirus (HPV) types 16 and 18 is closely associated with the development of cervical carcinoma. The transforming potential of these high-risk HPVs depends on the expression of the E6 and E7 early viral gene products. Since the expression of E6 and E7 is selectively maintained in premalignant and malignant cervical lesions these proteins are attractive candidates for immunotherapeutic and prophylactic strategies. Here we describe the construction, characterization and the *in vivo* immunotherapeutic potential of recombinant Semliki Forest virus (SFV) expressing the HPV16 E6 and E7 proteins (SFV-E6E7). Western-blot analysis and immunofluorescence staining demonstrated expression of E6 and E7 in BHK cells infected with SFV-E6E7. Immunization of mice with SFV-E6E7 resulted in an efficient *in vivo* priming of HPV-specific CTL activity. The induced CTLs lysed murine tumor cells transformed with the HPV16 genome and EL4 cells loaded with an immunodominant class-I-binding HPV E7 peptide. CTLs could reproducably be induced by immunization with three injections of as few as 10^5 infectious units of SFV-E6E7. Protection from tumor challenge was studied using the tumor cell line TC-1. Immunization with $5x10^6$ SFV-E6E7 particles protected 40% of the mice from tumor challenge. These results indicate that E6E7 expression by the efficient and safe recombinant SFV system represents a promising strategy for immunotherapy or immunoprophylaxis of cervical carcinoma.

Results

Production and titer determination of SFV particles

Recombinant SFV particles were produced in BHK cells by electroporation of recombinant and Helper 2 RNA into these cells. After 24 hr the medium containing the virus was removed from the cells and the virus particles were purified. Titers were determined by immunofluorescence using an antibody against SFV-nsP3 (replicase). This antibody was chosen because replicase is present in all cells infected with recombinant SFV. Thus, titers of different recombinant SFVs can be determined, independent of the inserted foreign gene(s). Typically, titers of unpurified virus were 10^9-10^{10} infectious units/ml. After purification titers were between 10^{10}-10^{11} infectious units/ml.

Western blot analysis of E6 and E7 expression

In order to verify that SFV-E6E7 induced expression of the recombinant E6 and E7 proteins, BHK cells were infected with SFV-E6E7 or SFV-LacZ serving as negative control. In Figure 1, Western blots of cell lysates probed with anti-HPV16 E6 (panel A) or anti-HPV16 E7 (panel B) are shown. Staining with the anti-E6 polyclonal antibody revealed a band with a M_r of approximately 17 kDa. Staining with the anti-E7 monoclonal antibody revealed a band with an apparent electrophoretic mobility of approximately 20 kDa. This M_r does not correspond to the calculated M_r (11 kDa) but is in agreement with other studies in which E7 was produced by eukaryotic as well as prokaryotic expression systems.[17-20]

Expression of HPV16 E6 and E7 in SFV-E6E7 infected cells

Expression of E6 and E7 was also analyzed by indirect immunofluorescence analysis of BHK cells infected with SFV-E6E7. A low particle-to-cell ratio was chosen such that not all cells in the wells would become infected, in order to visualize positive and negative cells within one microscopic field. As shown in Figure 2, a strong fluorescence of both E6 and E7 was found in infected cells. In general a bright staining of E6 was found in the perinucleus and cytoplasm while E7 was mainly found in the perinucleus. Previous studies demonstrated localization of the HPV18 E6 protein in the nuclear matrix and in non-nuclear membranes[21,22] and of HPV16 E7 in the nucleus.[18] However, it is very likely that differences in staining pattern may be influenced by the amounts of proteins produced and the vector used for expression of the proteins.[18]

HPV-specific CTLs induced by immunization of mice with SFV-E6E7

Mice were immunized s.c. and boosted twice (s.c. and i.p) with 10^6 purified SFV-E6E7, SFV-LacZ particles or buffer, as a control. CTL activity was determined one week after the last booster immunization. After 11 and 18 days of *in vitro* restimulation the resulting effector cells were tested for their cytolytic activity against 13-2 target cells. As show in Figure 3, a strong CTL activity was induced upon administration of SFV-E6E7 particles (Figures 3A and 3B, squares and diamonds), whereas no HPV-specific CTL activity was induced upon immunization with SFV-LacZ particles or PBS (Figures 3, triangles and crosses, respectively). The average level of cytolysis at day 11 (Figure 3A) increased slightly upon prolonged *in vitro* restimulation, i.e. 18 days culture (Figure 3B).

Since 13-2 cells only express the MHC class I H-$2D^b$- binding CTL epitope of HPV16 E7 peptide 49-57 (RAHYNIVTF),[9] CTL clones directed against other epitopes on E6 and E7 are not detected. We also tested CTL activity against C3 cells as target cells, i.e. cells that express the entire HPV16 genome. CTLs present after 11 days of - restimulation using C3 cells as stimulator cells, lysed 13-2 cells (Figure 4A) and C3 cells (Figure 4B) to the same extent. This result suggests that the HPV16 E7 peptide 49-57 is one of the dominant CTL epitope recognized by CTLs generated in C57BL/6 (H-2^b) mice upon immunization with E6 and E7.

This suggestion is supported by the observation that target cells loaded with HPV16 E7 49-57 were recognized and lysed to a very high level. Figure 5A shows CTL activity, induced in two mice immunized with 10^6 SFV-E6E7 particles, against EL4 cells loaded with the E7 49-57 peptide as targets. On the other hand, mice immunized with SFV-lacZ particles or PBS did not recognize peptide-loaded EL4 cells (Figure 5A, triangles and crosses, respectively). In addition, unloaded EL4 cells were not recognized and lysed by these CTLs (Figure 5B).

To determine the minimal effective dose of SFV-E6E7 particles, mice were immunized and boosted twice with 10^4, 10^5 or 10^6 particles per immunization. In

Figures 6A and 6B, the results of two separate experiments, each including two mice per injection dose, are given. In both experiments, immunization with 10^6 SFV-E6E7 particles (squares) but also with as few as 10^5 particles (diamonds), all mice developed an HPV-specific CTL response. Immunization with 10^4 particles (Figure 6, solid triangles), resulted in a low but detectable response in two out of four mice.

Antitumor responses induced by immunization of mice with SFV-E6E7 particles
To examine whether recombinant SFV particles could generate protective immunity against a subsequent tumor challenge, mice were immunized and boosted with SFV-E6E7 particles and challenged s.c. with TC-1 cells, tumor cells expressing HPV16 E6E7. Tumor inoculation studies performed before initiating these immunization studies revealed that s.c. inoculation of 2×10^4 TC-1 cells reproducably induced tumors within 2 to 4 weeks after inoculation in all mice tested (n=15). Figure 7 and 8 show combined results of two separate immunization studies. Control mice, injected with PBS (n=10) or SFV-LacZ particles (n=10) developed palpable tumors within 2 to 4 weeks after tumor cell inoculation (Figure 7, panels A and B, respectively; Figure 8, open circles and open squares, respectively). Immunization with 10^6 SFV-E6E7 particles (n=10) resulted in a delay in tumoronset in 50% the mice as compared to control mice, with one out of ten mice not developing a tumor (Figure 7, panel C; figure 8 diamonds). Upon immunization with a 5-fold higher dose of SFV-E6E7 particles two out of five mice did not develop a tumor (Figure 7, panel D; Figure 8, closed squares).

This example describes the construction, characterization and cellular immunotherapeutic potential of recombinant SFV particles encoding the early proteins E6 and E7 of HPV16. The ultimate aim of our studies is to develop an effective immunization strategy for the treatment and/or prevention of HPV-induced cervical carcinoma.

Immunization of mice with SFV particles encoding HPV16 E6 and E7 resulted in a HPV-specific CTL response. Three injections of as few as 10^4 SFV particles sufficed for the induction of a CTL response in 50% of the mice, while three immunizations with 10^5 SFV particles induced a HPV-specific CTL response in all mice tested. Increasing the dose to 10^6 SFV particles per injection resulted in a reproducible CTL response with a high level of specific tumor cell lysis. *In vitro* blocking experiments with antibodies against CD4 and CD8 revealed that the lytic activity was due to $CD8^+$ T cells, no inhibition was found with anti-CD4 antibodies (not shown). Tumor challenge experiments demonstrated that immunization with 10^6 SFV-E6E7 particles resulted in a delay in tumoronset while one of ten mice did not develop a tumor. Upon immunization with a 5-fold higher dose of SFV-E6E7 particles 40% of the mice did not develop a tumor.

In the last few years a number of peptide/protein-based or genetic immunization strategies have been described for the induction of HPV-specific CTL activity.[10-12] Major drawbacks associated with a peptide-based approach include the problem of MHC-polymorphism and the risk of inducing T cell tolerance rather than T cell activation. Due to the induction of specific T cell tolerance, vaccination with a tumor-specific peptide has been shown to result in an enhanced outgrowth of the tumor.[23] Immunization with larger proteins would overcome these problems, but requires efficient antigen delivery systems and/or safe adjuvants for efficient immune priming. Several groups have described the induction of HPV-specific CTL responses in mice upon immunization with recombinant vaccinia virus expressing HPV E6 or E7[24,25] or with syngeneic cells retrovirally transfected with the HPV E6 gene.[26] In a phase I/II trial involving eight patients with late stage cervical cancer, vaccination with recombinant vaccinia virus expressing HPV18 E6 and E7 induced HPV-specific CTLs in one of three evaluable patients.[27] Potential drawbacks associated with the use of viral vector systems are immune responses against viral proteins in pre-immune patients (vaccinia virus) or integration of recombinant genes into the host cell genome (retrovirus). Especially, when the recombinant virus encodes oncoproteins such as HPV E6 or E7, the risk of integration into the host cell genome is a point of major concern.

We have chosen for the SFV expression system which, apart from its transfection efficiency and high biosafety, would appear to be especially suited to safely induce cellular immune responses against oncoproteins such as HPV16 E6 and E7. Firstly, SFV is an RNA virus replicating in the cell cytosol; therefore, there is no risk of integration of the E6 and E7 genes in the cellular genome. Moreover, SFV infection is cytolytic by apoptosis.[13,28] Therefore no genetic information of E6 and E7 will persist for more than one week after injection. In addition, no other vector proteins are produced, besides small amounts of viral replicase. Berglund *et al.* demonstrated that immune responses against the vector itself did not inhibit boost responses by subsequent immunizations with the same vector .[29]

Recognition by the immune system of virally-infected cells or tumor cells occurs via virus- or tumor-specific antigenic peptides presented in the context of MHC class I molecules. Infection of cells with recombinant SFV particles results in the production of the recombinant protein within the cytoplasm permitting presentation of the recombinant protein via the conventional MHC class I presentation route. However, for the induction of tumor- or virus-specific CTLs, antigen presentation has to be accompanied by costimulatory signalling. Costimulatory molecules are confined to professional antigen-presenting cells (APCs). Therefore, the CTL response induced upon immunization with SFV-E6E7 particles may occur through transfection of APCs *in vivo*. Alternatively, APCs may take up residues of other cells that have been transfected in a process of cross-priming. The uptake of debris from infected cells by APCs is expected to be very efficient since an SFV infection induces apoptotic cell death.[13,28] Upon uptake of infected-cell material (exogenous antigen) the recombinant protein will be processed and presented by MHC class II molecules thereby activating CD4+ T-helper cells. In addition, dendritic cells and macrophages are able to present exogenous antigen in the context of MHC class I molecules for presentation to and activation of CD8+ T cells.[30] Thus, both arms of the cellular immune system, essential for the induction of an optimal immune response will be activated upon administration of recombinant SFV particles, thereby eliciting a potent CTL response. Moreover, SFV immunization will introduce both class I and class II antigenic epitopes into one and the same the APC which has recently been demonstrated to be required for a full activation of APCs.[31-34] As demonstrated by Zhou *et al.*[35] immunization of mice with SFV particles encoding for the nucleoprotein of influenza virus not only induces influenza-specific CTL activity, but also an nucleoprotein-specific antibody response. This observation supports the hypothesis of cross-priming and indirect presentation of antigenic peptides.

In conclusion, we demonstrated that immunization of mice with recombinant SFV-E6E7 particles induces a potent CTL response against HPV-transformed tumor cells. This promising result, combined with studies showing the high efficacy of the SFV system for priming the immune system of mice as well as primates,[29,35,36] and the recent development of the extremely safe two-helper system[37] provide the essential steps towards the design of an effective immunization strategy for the treatment and prophylaxis of HPV-induced cervical carcinoma.

Materials and methods

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). The cells were grown in GMEM (Life Technologies, Paisley, UK) containing 5% fetal calf serum (PAA laboratories, Linz, Austria). C3 cells, 13-2 cells and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57BL/6 (H-2^b) embryonic cells transfected with a plasmid containing the complete HPV16 genome. The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF) (R. Offringa, personal communication). The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-*ras*[25]. EL4 cells were kindly provided by Dr. L. Leserman (Centre d'Immunologie de Marseille-Luminy, France). C3, 13-2, TC-1 and EL4 cells were grown in IMDM (Life Technologies) supplemented with 10% fetal calf serum. Both media contained penicillin and streptomycin (Life Technologies; 100 U/ml and 100 µg/ml, respectively).

Mice

Specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunization protocols.

Peptide

The HPV16 H-2D^b binding E7 peptide RAHYNIVTF (residue 49-57) was synthesized and purified by Dr. J.W. Drijfhout (Academic Hospital Leiden, The Netherlands). The peptide was analyzed by reverse phase HPLC and found to be over 90% pure.

Cloning of HPV16 E6 and E7 in pSFV3

pSFV-Helper 2[16] and pSFV3[15] were kindly provided by Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden). The HPV16 E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7,[38] which was kindly provided by Dr. J. Ter Schegget (Free University, Amsterdam, The Netherlands). In this plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. Amplification of the E6E7 tandem gene was done by PCR using the following primers, written in 5' to 3' direction: GACGGATCCAAAGAGAACTCCAAT G (E6 forward) and GAGAATTCGGATCCGCCATGGTAGATTAT (E7 reverse). The PCR product was digested with BamHI and cloned into the BamHI site of pGEM7Zf+. After sequence confirmation, the E6E7 fragment was cloned into the unique BamHI site of pSFV3, producing pSFV3-E6E7.

Production and purification of recombinant SFV particles

pSFV3-LacZ[15] was a kind gift from Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden). The pSFV3-E6E7, pSFV3-LacZ and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit and linearized by digestion with SpeI (Life Technologies). RNA was synthesized from the linearized DNA by *in vitro* transcription using SP6 RNA polymerase (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Capping analogue was obtained from Life Technologies. Fifteen μg SFV3-E6E7 or SFV3-LacZ and 7.5 μg SFV-Helper 2 RNA were admixed and cotransfected into $8x10^6$ BHK cells in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser^{R}II (two pulses of 850 V/ 25 μF; Biorad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured for 36 hr at 37°C and 5% CO_2. The medium, containing the SFV-E6E7 or SFV-LacZ particles was centrifuged twice in a JA 20 rotor (Beckman, St. Paul, MN, USA) at 1800 rpm (i.e. 40,000xg at r_{max}) to remove cells and cellular debris.

The SFV particles were purified on a discontinous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) and 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1mM EDTA, pH 7.4)). Virus was collected from the interface. Sucrose was removed from the virus solution by overnight dialysis against TNE-buffer. The virus suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N_2 and stored in aliquots at -80°C.

Before use, SFV particles were incubated with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated spike proteins. Subsequently, α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ ml; Sigma Chemical Co.).

Titer determination of SFV particles

Recombinant SFV particles were titrated by serial dilution on monolayers of BHK cells. After infection and overnight incubation the cells were fixed for 10 minutes in 10% acetone and stained using a polyclonal rabbit anti-replicase (nsP3) antibody (a kind gift from Dr T. Ahola, Biocentre Viiki, Helsinki, Finland) as primary antibody and FITC-labelled goat-anti-rabbit IgG as a secondary antibody (Southern Biotech. Ass., Birmingham, AL, USA). Positive cells were counted and the titer was determined after correcting for the dilution factor and the dilution caused by the activation and the volume of particles added.

Analysis of E6 and E7 expression by Western blotting

BHK cells were infected with SFV-E6E7 particles or as a control with SFV-LacZ particles. After overnight incubation, the cells were harvested and lysed in lysis buffer (50 mM Tris.Cl, 5 mM EDTA, 150 mM NaCl, 0,5% Triton X-100, pH 7.4). Cell-free extracts were analyzed by SDS-PAGE. The proteins were blotted onto PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA) and E6 and E7 were detected with a polyclonal rabbit-anti-HPV16 E6 antibody (a kind gift from Dr. I. Jochmus, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and a monoclonal mouse-anti-HPV16 E7 antibody (Zymed Lab. Inc. South San Francisco, CA, USA), respectively. After incubation with alkaline phosphatase-linked secondary antibodies the blots were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.).

Indirect immunofluorescence analysis of E6 and E7 in SFV-E6E7 infected cells

In an 8-well culture chamber slide (Life Technologies) a monolayer of BHK cells was infected with SFV-E6E7. Fixation of the cells and staining was done as described for the immunofluorescence with anti-replicase, except for the used antibodies. As primary antibodies, anti-HPV16 E6 or anti-HPV16 E7, as mentioned above, were used. The secondary antibodies were FITC-labeled anti-rabbit IgG and anti-mouse IgG, respectively (Southern Biotechn. Ass., Birmingham, AL, USA).

Immunizations

Mice were immunized subcutaneously (s.c.), intraperitoneally (i.p.) or intravenously (i.v.) and boosted twice with a 2-week interval, with 10^4 to $5x10^6$ SFV-E6E7 particles. As negative controls, mice were injected with equal doses of SFV-LacZ particles or PBS.

CTL assay

Seven to 21 days after the last booster immunization, spleen cells were isolated and cocultured with irradiated (100 Gy) C3 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. After one and two weeks in culture, cells were harvested and restimulated with irradiated naive spleen cells (30 Gy) and irradiated C3 cells in a ratio of 2:5:0.1 in 24-well plates in the presence of 4 IU of recombinant hIL2/ml (Strathmann Biotech GMBH, Hamburg, Germany). Five days after the first and/or second restimulation, cells were harvested and a CTL assay was performed by a standard 4 hr ^{51}Cr release assay in triplicate determinations. Target cells were labeled for 1 h with 3.7 MBq ^{51}Cr/10^6 cells in 100 µl medium (^{51}Cr was from Amersham, London, UK). EL4 target cells were loaded with the HPV16 E7 49-57 (RAHYNIVTF) peptide by a 1 hr incubation of the cells in the presence of 15 µg/ml of peptide in 100 µl of culture medium before labeling the cells with ^{51}Cr. The mean percentage of specific ^{51}Cr release of triplicate wells was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

Tumor challenge experiments

Mice were immunized and boosted as described above with 10^6 to 5x10^6 SFV-E6E7 particles, SFV-LacZ particles or PBS. One week after the last booster immunization the mice were challenged s.c. with 2x10^4 TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Life Technologies). Tumor measurements were always done by the same skilled technician. At a tumorvolume of approximately 1000 mm^3, the mice were sacrificed.

Example 2

We further generated two recombinant SFV plasmids that contain a translational enhancer. One plasmid encodes a fusion protein of E6 and E7 by inserting one base pair between E6 and E7 and by changing the stop codon of E6, this plasmid is named pSFV3-eE6,7 (Figures 9 and 19). In the other plasmid the translational enhancer is placed in front of the original E6E7 construct, pSFV3-eE6E7.

Western blot analysis of protein expression

To verify that SFV-eE6,7 induced expression of a recombinant fusion protein of E6 and E7 while SFV-E6E7 induces expression of the separate E6 and E7 proteins, lysates of cells infected with SFV-E6E7 or SFV-eE6,7 were compared by Western blot analysis. In addition, lysates of cells infected with the construct in which the translation enhancer was cloned in front of the original E6E7 construct, i.e. SFV-eE6E7, were analysed.

In Figure 10, Western blots probed with anti-HPV16 E6 or anti-HPV16 E7 are shown. Staining with both the anti-E6 and the anti-E7 antibody revealed three prominent bands in lysates from cells infected with SFV-eE6,7 (lanes 3 and 7), while no or very little E6 and E7 could be demonstrated upon infection with SFV-E6E7 (lanes 1 and 5). However, it should be noted that the procedure (amount of material and staining time) used for demonstration of the highly expressed fusion protein by Western blotting is not optimal for demonstration of the relatively low expression of E6 and E7 in SFV-E6E7 infected cell. In a previous study expression of E6 and E7 could be demonstrated in cells infected with SFV-E6E7 using more material and a longer staining time.

The three major bands observed in the SFV-eE6,7 lysates had apparent electrophoretic mobilities of approximately 26 kDa, 36 kDa and 44 kDa, respectively. The 26 kDa band represents the fusion protein of E6 and E7 (17 kDa and 11 kDa, respectively). The bands of 36 kDa and 44 kDa however, do not correspond to the calculated M_r's of dimeric and trimeric complexes of the fusion protein. Nonetheless, since both bands stain positive with the anti-E6 antibody as well as with the anti-E7 antibody, the bands reflect a protein complex composed of both E6 and E7. In this regards it should be noted that, others and we have demonstrated, that the M_r of recombinant produced E7 protein (Figure 10, lane 8) does not correspond to the calculated M_r (11 kDa). Similarly, the apparent M_r's of the bands may not reflect the actual M_r's of the fusion proteins.

Staining of the lysate from cells infected with SFV-eE6,E7 with the anti-E6 antibody revealed two bands of approximately 22 kDa and 32 kDa (Figure 10, lane 2). Staining of this lane with the anti-E7 antibody did not reveal a band (Figure 10; lane 6) demonstrating that, as expected, only the E6 protein was translated in an enhanced fashion. The 22 kDa band observed in the ant-E6 blot is slightly higher than the calculated Mr of E6, i.e.17 kDa. The 32 kDa band might represent a dimeric complex of E6.

Analysis of E6 and E7 expression by pulse-labelling

Production and stability of E6, E7 and the fusion protein E6,7 by BHK cells transfected with SFV particles was analysed by pulse-chase-labelling of the cells with ^{35}S-methionine/cysteine. As shown in Figure 11 (lane 6), infection of BHK cells with SFV-eE6,7 particles and radiolabelling for one hour, resulted in three prominently labelled bands of the E6,7 fusion protein. These bands correspond to the bands revealed on the Western blots. Although it may seem as if the 44 kDa band is also present in the control lysates, closer examination reveals that this upper fusion protein band in lanes 6-9 runs slightly lower than the band in lanes 1-5. The bands of 36 and 46 kDa are still present after a 6- and 16-h chase period. Even after a 40-h chase period both bands, although less bright, are visible. The short exposure time that sufficed to visualise the enhanced fusion proteins could not reveal the bands of the E6 and E7 proteins produced upon infection with SFV-E6E7, either following a 6-h chase period (Figure 11; lane 2) or directly upon labelling (not shown). Previously, we demonstrated that a longer exposure time of the film is needed to visualise these proteins.

Autoradiography of lysates from cells infected with SFV-eE6E7 directly after labelling (Figure 11, lane 3) revealed the same bands as those observed by Western blot analysis, i.e. a 22 and a 32 kDa band. However, in contrast to the enhanced fusion protein, within a 6-h chase period most of the E6 protein was degraded. After 16 h the protein was degraded almost completely.

HPV-specific CTLs induced by immunisation of mice with SFV-eE6,7 and SFV-E6E7

Mice were immunised s.c. and boosted twice (s.c. and i.p) with 10^6 purified SFV-E6E7, SFV-eE6,7, SFV-eE6E7, SFV-LacZ particles or buffer, as a control. CTL activity was determined one week after the last booster immunisation. After 7 days (Figure 12 A) and 14 days (Figure 12 B) of *in vitro* restimulation the resulting effector cells were tested for their cytolytic activity against 13-2 target cells and C3 target cells. Similar levels of cytolysis were induced against both cell lines. As show in Figure 12 , spleen cells isolated from mice immunised with 10^6 but also with as few as 10^5 SFV-eE6,7 particles already displayed a high level of cytolysis in the short-term restimulation protocol (i.e. 7 days; Figure 12 A). Upon immunisation with 10^6 SFV-E6,E7, significant levels of CTL activity could be determined after long-term restimulation only (Figure 12 B), short-term restimulation resulted in a very low level of CTL activity. Upon immunisation with 10^5 SFV-E6E7 no CTL activity was detectable. Immunisation with SFV-eE6,E7 did not induce detectable levels of CTL activity against 13-2 cells nor against C3 target cells, that express the entire HPV16 E6E7 genome (not shown).

Next, the level and maintenance of CTL activity induced upon administration of higher dosages of SFV-eE6,7 particles were determined. Mice were immunised with 1, 2.5 or 5 x10^6 SFV particles and CTL activity was determined 18 days and 8 weeks after the last booster immunisation. As shown in Figure 13 , the level of CTL activity induced with 10^6 SFV-eE6,7 is presumably the maximal level of lysis that can be reached and detected in the bulk CTL ^{51}Cr-release assay as immunisation with 2.5 and 5 x 10^6 SFV-eE6,7 did not increase the percentage of specific lysis. Importantly, 8 weeks after the last booster immunisation levels of cytolysis were as high as 18 days after the last booster (Figure 13 B and Figure 13 A, respectively).

For this bulk CTL assay, spleen cells are restimulated *in vitro* for several days resulting in proliferation of CTL precursors. Therefore this assay is not a reliable assay to determine the actual frequency of CTL precursors that has been induced *in vivo*. To evaluate the number of CTL precursors an HPV16 IFN-ã Elispot assay was performed. As demonstrated in Table 1, the number of CTL precursors 18 days after injection of 1, 2.5 and 5 x 10^6 SFV-eE6,7 particles was within the range of 1 in 1780 to 1 in 6600 total spleen cells. Since approximately 8% of the C57Bl/6 spleen cells are CD8+ T cells this means that 1 in 140 to 1 in 530 CD8-positive splenic T cells is HPV specific. Eight weeks after the last booster immunisation the level ranged between 1 in 430 to 1 in 1090 $CD8^+$ T cells. Although no firm conclusions can be drawn from these numbers, since each dose and time point represents a single mouse, no correlation was observed between the dose injected and the level of specific CTLs.

In previous immunisation protocols mice were always immunised three times (i.e. one primary immunisation followed by two booster immunisations). In order to determine the number of immunisations needed to induce a long-term response, we immunised mice once or twice and determined the level of CTL activity at 10 days, 1 month or three months after the last immunisation.
Figure 14A shows that a single immunisation of 2.5x10^6 SFV-eE6,7 particles induces a significant level of cytolysis at 10 days after immunisation (squares). This level gradually decreases in the next three months (1 month: circles; three months: triangles). However, a single boost suffices to induce a significant CTL response upto 3 months after the booster immunisation (Figure 14B, triangles) which was as high as the response after 1 month (Figure 14B, circles).

Finally, the immunisation route was varied. Mice were immunised s.c, i.p. or i.v. with 1x10^6 SFV-eE6,7 particles. The bulk CTL assay shows that the levels of specific lysis by spleen cells isolated from the i.v. immunised mice (n=3) are slightly higher (at E:T ratio of 3 already near-maximal lysis) than those of mice immunised s.c. (n=2) or i.p. (n=3) (Figure 15, upper three panels). Separately, using the same spleen cells, CTL precursor frequencies were determined using HPV 16 E7 specific MHC class I tetramers. In the lower panels of Figure 15, the percentages of tetramer$^+$/CD8$^+$ T cells are given. The bars correspond to the CTL data in the upper panels. The relatively higher level of specific lysis observed upon i.v. immunisation is also reflected in the number of tetramer$^+$/CD8$^+$ T cells.

Tumour challenge and rechallenge upon immunisation of mice with SFV-eE6,7 particles

To examine whether recombinant SFV particles could generate protective immunity against a subsequent tumour challenge, mice were immunised and boosted with SFV-eE6,7 particles and challenged s.c. with TC-1 cells, tumour cells expressing HPV16 E6E7. Figure 16 shows combined results of two separate immunisation studies. Control mice, injected with PBS (n=10) or SFV-LacZ particles (n=10) developed palpable tumours within 2 to 4 weeks after tumour cell inoculation. In a previous study we demonstrated that immunisation with 5x10^6 SFV-E6E7 particles resulted in a partial tumour protection, i.e. two of five mice did not develop a tumour. Here we demonstrate, that immunisation with 10^6 SFV-eE6,7 particles protects 9 out of ten mice from developing a tumour (Figure 16). Immunisation with a 5-fold higher dose (5x10^6 particles) protects 4 out of 5 mice (table 2).

To determine if long-term protection is induced, mice that did not develop a tumour were rechallenged s.c. with 2x10^4 tumour cells at week 25 (exp. 1) or at week 13 (exp. 2) after the initial tumour challenge.

As shown in Table 2, all mice immunised with 5x10^6 SFV-eE6,7 that did not develop a tumour at the initial tumour challenge were protected against the second tumour challenge 13 weeks later. Of the mice immunised with 10^6 SFV-eE6,7 50% and 60% did not develop a tumour upon a second tumour challenge at week 25 and week 13, respectively.

Tumour treatment upon immunisation with SFV-eE6,7 particles

The promising results obtained in the tumour challenge experiments as described above prompted us to determine the efficacy of SFV-eE6,7 immunisation in a tumour treatment setting. Mice were inoculated s.c with 2x10^4 TC-1 cells. At several time points after tumour inoculation, mice were immunised s.c. with 5x10^6 SFV-eE6,7 particles, SFV-LacZ or PBS. In Figure 17 the tumor volumes of individual mice of two separate experiments are shown. Figure 18 shows the combined results of these experiments as percentages of tumour free mice in time. Control and SFV-LacZ injected mice developed a tumour within 2 weeks after tumour inoculation. All mice immunised with SFV-eE6,7 particles on days 2, 7 and 14 after tumour inoculation were tumour free at day 100 after tumour inoculation (Figure 17, panel C). In 4 of 7 mice of this group a very small tumour was palpable at day 14 after tumour inoculation (Figure 17). These tumour nodules disappeared within 7 days and all mice remained tumour free. In the second group of mice, immunised with SFV-eE6,7 particles on days 7, 14 and 21 after tumour inoculation, all mice (n=14) in both experiments developed a small palpable tumour on day 14. In 12 of 14 mice these nodules had disappeared on day 21. Ultimately, 9 of 14 mice remained tumour free (Figure 17). Finally, in one group of mice immunisation was initiated as late as day 14 after tumour inoculation. As shown in Figure 17, 3 of 7 mice cleared the initial tumours. One of these mice cleared a tumour with a volume of 650 mm^3 on day 20. One mouse again developed a tumour as late as 60 days after tumour inoculation.

Tumour challenge and rechallenge upon s.c. immunisation with SFV-eE6,7 particles

In one of the experiments described above, tumour-free mice were re-challenged with TC-1 cells without additional immunisation, 13 weeks after the initial tumour inoculation. Since none of the control mice were tumour-free after 13 weeks, 4 control mice were included in the experiment at the time of the second tumour inoculation. As shown in figure 20, all tumour-free mice immunised at days 7, 14 and 21 after the initial tumour inoculation remained tumour-free upon a second tumour challenge. In the group of mice immunised on days 2, 7 and 14 only one of seven mice developed a tumour after the second tumour inoculation.

Tumour treatment upon intravenous immunisation with SFV-eE6,7 particles

Elispot analysis and tetramer staining demonstrated that upon intravenous immunisation higher numbers of precursor CTLs are induced than after s.c. and i.p. immunisation. We therefore performed tumour therapy studies in which mice were immunised intravenously. The experiments were performed similar to the experiments described above. As shown in figure 21, all mice immunised with 5x10^6

SFV-eE6,7 particles on days 7, 14 and 21 after tumour inoculation, remained tumour-free. Even when immunisation was initiated 14 days after tumour inoculation, a time-point at which all mice have developed a palpable tumour, all tumours regressed. Ultimately, 4 of 7 mice eradicated the tumour completely and remained tumour-free thereafter.

Tumour treatment upon intravenous immunisation with as few as 5x10^4 SFV-eE6,7 particles

To determine the minimal effective dose, mice were immunised with decreasing amounts of SFV-eE6,7 particles at days 7, 14 and 21 after tumour inoculation. As demonstrated in figure 22, all mice immunised with 5x10^5 SFV-eE6,7 particles and six of seven mice immunised with as few as 5x10^4 SFV-eE6,7 particles cleared the tumour and remained tumour-free upto 10 weeks after tumour inoculation.

Materials and methods

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). The cells were grown in GMEM (Life Technologies, Paisley, UK) containing 5% foetal calf serum (PAA laboratories, Linz, Austria). C3 cells, 13-2 cells and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57BL/6 (H-2^b) embryonic cells transfected with a plasmid containing the complete HPV16 genome. The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF) (R. Offringa, personal communication). The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-*ras*[25]. C3, 13-2 and TC-1 cells were grown in IMDM (Life Technologies) supplemented with 10% foetal calf serum. Both media contained penicillin and streptomycin (Life Technologies; 100 U/ml and 100 µg/ml, respectively).

Mice

Specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunisation protocols.

Construction of pSFV3-E6E7, pSFV3-eE6,7 and pSFV-eE6E7

pSFV-Helper 2[16] and pSFV3[15] were kindly provided by Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden). The HPV16 E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7,[38], which was kindly, provided by Dr. J. Ter Schegget (Free University, Amsterdam, The Netherlands). In this plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. Amplification of the E6E7 tandem gene was done by PCR. The PCR product was digested with BamHI and cloned into the BamHI site of pGEM7Zf+. After sequence confirmation, the E6E7 fragment was cloned into the unique BamHI site of pSFV3, producing pSFV3-E6E7.

The plasmid pSFV3-eE6,7 was generated to express high levels of a fusion protein of HPV16 E6 and E7 by including a translational enhancer. The construction is depicted in Figure 17 and as described as follows.

Out of the pSFV3-E6E7 the E6 sequence was modified with an NcoI site at the 5' end and an EcoRI site at the 3' end. The E7 sequence was modified with an EcoRI site at the 5' end and a BamHI site at the 3' end by PCR.

The 5' end of the capsid gene of SFV coding for the first 34 amino acid residues has been shown to contain a translational enhancer. This enhancer was cloned in a pSFV-helper-S1 construct by Smerdou and Liljestrom (J.Virol. 73, 1092-1098, 1999). In addition they inserted the sequence of foot-and-mouse disease virus (FMDV) 2A autoprotease (17 amino acids) in frame between the translational enhancer and p62 (SFV envelope protein) in order to provide cleavage between the proteins. We synthesized the sequence containing the translational enhancer and the FMDV A2 autoprotease from pSFV-helper-S1, and by PCR BamHI and NcoI restriction sites were generated at the 5' and 3' end, respectively. The enh-FMDV A2 protease-, E6- and E7 fragments were cloned into the BamHI site of pSFV3, producing pSFV3-eE6,7.

In the original plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. In pSFV3-eE6,7 one base pair is inserted between E6 and E7 and the stop codon TAA of E6 is changed in GAA. Thus, in pSFV3-eE6,7 the sequence encoding E6 and E7 is in frame, expressing a fusion product of E6 and E7.

The construction of pSFV3-eE6E7 was done by cloning the intact E6E7 fragment and the translational enhancer-FMDV A2 autoprotease fragment in pSFV3-eE6E7. Since E6 and E7 are not in frame it is to be expected that this plasmid encodes E6 in an enhanced fashion while translation of E7 is not enhanced.

The inserts encoding eE6E7 or eE6,7 were sequenced to verify that no modifications had been generated during the PCR

Production and purification of recombinant SFV particles

pSFV3-LacZ[15] was a kind gift from Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden). The pSFV3-E6E7, pSFV3-eE6E7, pSFV3-eE6,7, pSFV3-LacZ and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit and linearised by digestion with SpeI (Life Technologies). RNA was synthesised from the linearised DNA by *in vitro* transcription using SP6 RNA polymerase (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Capping analogue was obtained from Life Technologies. Fifteen μg SFV3-E6E7 or SFV3-LacZ and 7.5 μg SFV-Helper 2 RNA were admixed and cotransfected into $8x10^6$ BHK cells in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser^{R}II (two pulses of 850 V/ 25 μF; Biorad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured for 36 hr at 37°C and 5% CO_2. The medium, containing the SFV-E6E7 or SFV-LacZ particles was centrifuged twice in a JA 20 rotor (Beckman, St. Paul, MN, USA) at 1800 rpm (i.e. 40,000xg at r_{max}) to remove cells and cellular debris.

The SFV particles were purified on a discontinous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) and 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1mM EDTA, pH 7.4)). Virus was collected from the interface. Sucrose was removed from the virus solution by overnight dialysis against TNE-buffer. The virus suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N_2 and stored in aliquots at -80°C.

Before use, SFV particles were incubated with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated spike proteins. Subsequently, α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ ml; Sigma Chemical Co.).

Titer determination of SFV particles

Recombinant SFV particles were titrated by serial dilution on monolayers of BHK cells. After infection and overnight incubation the cells were fixed for 10 minutes in 10% acetone and stained using a polyclonal rabbit anti-replicase (nsP3) antibody (a kind gift from Dr T. Ahola, Biocentre Viiki, Helsinki, Finland) as primary antibody and FITC-labelled goat-anti-rabbit IgG as a secondary antibody (Southern Biotech. Ass., Birmingham, AL, USA). Positive cells were counted and the titer was determined after correcting for the dilution factor and the dilution caused by the activation and the volume of particles added.

Analysis of E6 and E7 expression by Western blotting

BHK cells were infected with SFV-E6E7, SFV-eE6,7 or SFV-eE6E7 particles. After overnight incubation, the cells were harvested and lysed in lysis buffer (50 mM Tris.Cl, 5 mM EDTA, 150 mM NaCl, 0,5% Triton X-100, pH 7.4). Cell-free extracts were analysed by SDS-PAGE. The proteins were blotted onto PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA) and E6 and E7 were detected with a polyclonal rabbit-anti-HPV16 E6 antibody (a kind gift from Dr. I. Jochmus, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and a monoclonal mouse-anti-HPV16 E7 antibody (Zymed Lab. Inc. South San Francisco, CA, USA), respectively. After incubation with alkaline phosphatase-linked secondary antibodies the blots were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.).

Analysis of E6 and E7 expression by pulse-labelling

For pulse-labelling, BHK cells were infected with SFV-E6E7, SFV-eE6,E7 or SFV-eE6,7 particles. After 6 hr, the medium was removed, the plates were washed three times with phosphate-buffered saline (PBS) and the cells were cultured for an additional 30 min with methionine- and cysteine-free DMEM (ICN Biomedicals). At this time point ^{35}S-methionine/cysteine (0.37 MBq/well; Amersham) was added to the cultures. After one hr the wells were washed free from radioisotope and harvested directely or culture for an additional 6, 16 or 40 h before harvesting. At these time points the cells were washed with PBS (4^{o}C), harvested by scraping and resuspended in lysis buffer containing 0.2 mM phenyl-methane-sulphonyl-fluoride. After centrifugation the supernants of the cell lysates were analysed by SDS/PAGE and autoradiography.

Immunizations

Mice were immunised subcutaneously (s.c.), intraperitoneally (i.p.) or intravenously (i.v.) with 10^4 to $5x10^6$ recombinant SFV particles, followed by one or two booster immunisation with a two-week interval or not boosted. As negative controls, mice were injected with equal doses of SFV-LacZ particles or PBS.

CTL assay

Seven days to 3 months after the last (booster) immunisation, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. After one week in culture, cells were harvested and a CTL assay was performed by a standard 4 hr ^{51}Cr release assay in triplicate determinations. Target cells were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 100 μl medium (^{51}Cr was from Amersham, London, UK. The mean percentage of specific ^{51}Cr-release of triplicate wells was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

Initially, CTL analysis was also performed after an additional period of *in vitro* stimulation. For these experiments spleen cells cultured for one week, as described above, were harvested and restimulated with irradiated naive spleen cells (30 Gy) and irradiated C3 cells in a ratio of 2:5:0.1 in 24-well plates in the presence of 4 IU of recombinant hIL2/ml (Strathmann Biotech GMBH, Hamburg, Germany). Five days after restimulation, cells were harvested and a ^{51}Cr-release assay was prerformed as described above.

Precursor CTL frequency determination by IFN-alpha Elispot analysis

Elispot analysis was done essentially according to the method described by Miyahira et al (J Immunol. Methods 181, 45054, 1995). In short, serially diluted, known numbers of freshly isolated spleen cells were plated into wells (96-well high affinity plates, Greiner) that had been coated overnight with purified anti-mouse-IFN-*alpha* mAb (Pharmingen, CA). Subsequently, 13-2 cells cells (only expressing the HPV16

$E7_{49-57}$ CTL epitope) were added for *in vitro* restimulation using effector to stimulator cell ratio's of 1:1 to 4:1. In addition, spleen cells were cultured without stimulator cells as controls to determine antigen-independent IFN-*alpha* secretion. After overnight incubation the wells were washed extensively and incubated with biotinylated anti-mouse IFN-*ã* mAb (Pharmingen, CA). After a 1-h incubation at 37°C, the plates were washed and strepavidine-alkaline phosphatase was added. After 1 h incubation at 37°C the plates were washed and the spots were developed by adding the substrate BCIP (Sigma) in agarose. After overnight incubation at 4°C the number of spots were determined using a stereomicroscope.

Precursor CTL frequency determination using HPV-specific MHC class I tetramers

Pycoerythrin (PE)-labeled HPV16 $E7_{49-57}$ MHC class I tetramers were kindly provided by Dr. T.Schumacher (NKI, Amsterdam, The Netherlands). Freshly isolated spleen cells were stained with PE-labeled HPV16 $E7_{49-57}$ tetramer and FITC-anti-CD8 (Pharmingen) for 20 min on ice followed by extensive washes with PBS containing BSA (0.5%) and NaN_3 (0.02%). Before FACS analysis, propidium iodide (PI) was added. Small lymphocytes were gated by forward and side scatter profiling.

Tumour challenge experiments

Mice were immunised and boosted as described above with 10^6 to $5x10^6$ SFV-E6E7, SFV-eE6,7 particles, SFV-LacZ particles or PBS. One week after the last booster immunisation the mice were challenged s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Life Technologies). Tumour measurements were always done by the same skilled technician. At a tumorvolume of approximately 1000 mm^3, the mice were sacrificed.

Tumour treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Life Technologies). At several time points after tumour inoculation mice were immunised subcutaneously or intravenously with $5x10^4$ to

$5x10^6$ SFV-eE6,7 particles. Mice were immunised on days 2, 7 and 14 after tumour inoculation, or on days 7, 14 and 21 after inoculation or finally on days 14, 21 and 28 after inoculation. In addition control groups were included that were immunised with either PBS or 5x106 SFV-LacZ particles on days 2, 7 and 14 after tumour inoculation. Tumour measurements were always done by the same skilled technician. At a tumorvolume of approximately 1000 mm^3, the mice were sacrificed.

Table 1

Precursor CTL frequency in SFV-eE6,7 immunised mice as determined by IFN-gamma Elispot assay

Immunisation	Dose	Evaluation time point	pCTL frequency total spleen[1]	pCTL frequency in CD8$^+$ T cells[2]
SFV-eE6,7	$1x10^6$	18 days	1 in 6557	1 in 524
"	$2,5x10^6$	"	1 in 1785	I in 143
"	$5x10^6$	"	1 in 4081	1 in 326
"	$1x10^6$	8 weeks	1 in 5381	1 in 430
"	$2.5x10^6$	"	1 in 13636	1 in 1090
"	$5x10^6$	"	1 in 7692	1 in 615
SFV-LacZ	$5x10^6$	18 days and 8 weeks	0 in $4x10^5$	-
PBS		"	0 in $4x10^5$	-

Mice were immunised s.c. and boosted twice (s.c. and i.p) with 1, 2.5 or $5x10^6$ *SFV-eE6,7 or with* $5x10^6$ *SFV-LacZ or PBS as controls.*

[1] *Spleen cells were isolated 18 days or 8 weeks after the last booster immunisation and the frequency of precursor CTLs was determined by INF-gamma Elispot assay upon overnight in vitro stimulation with 13-2 cells expressing HPV16-E7 49-57 (MHC class I epitope).*

[2] *Calculated frequency using a CD8 frequency of 8% of the total spleen.*

Table 2

Protection from growth of TC-1 tumour cells in SFV-eE6,7 immunised mice upon tumour challenge and rechallenge.

Immunisation[1]	Number of tumour-free mice after 1st tumour challenge/ total number of mice[2]	Number of tumour-free mice after 2nd tumour challenge/ total number of mice[3]
Exp. 1 *Tumour challenge day 0, tumour rechallenge week 25*		
SFV-eE6,7 [1x10^6]	4/5	2/4
SFV-LacZ [1x10^6]	0/5	-
PBS	0/5	0/3[4]
Exp. 2 *Tumour challenge day 0, tumour rechallenge week 13*		
SFV-eE6,7 [1x10^6]	5/5	3/5
SFV-eE6,7 [5x10^6]	4/5	4/4
SFV-LacZ [5x10^6]	0/5	-
PBS	0/5	0/3[4]

[1] *Mice were immunised s.c. and boosted twice (s.c. and i.p.) with 1x10^6 or 5x10^6 SFV-LacZ particles or SFV-eE6,7 particles or PBS. One week after the last booster immunisation, 2x10^4 TC-1 tumour cells were inoculated s.c. in the neck.*

[2] *Tumor growth was monitored twice weekly. Shown are the number of tumour-free mice per total number of mice per group.*

[3] *Mice that remained tumour-free were subsequently rechallenged with* $2x10^4$ *TC-1 cells. In the* 1st *experiment in week 25, in the* 2nd *experiment in week 13 after the first tumour challenge.*

[4] *In the rechallenge experiments three age-matched control mice were included.*

References

1 Zur Hausen H. Viruses in human cancer. *Science* 1991; **254**: 1167-1173.

2 Münger K, Scheffner M, Huibregtse JM, Howley PM. Interactions of HPV E6 and E7 with tumor suppressor gene products. *Cancer Surv* 1992; **12**: 197-217.

3 Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 1990; **248:** 76-79.

4 Pei XF. The human papillomavirus E6/E7 genes induce discordant changes in the expression of cell growth regulatory proteins. *Carcinogenesis* 1996; **17:** 1395-1401.

5 Jones DL, Münger K. Interactions of human papillomavirus E7 protein with cell cycle regulators. *Seminars Cancer Biol* 1996; **7:** 327-337.

6 Gulliver GA, Herber RL, Liem A, Lambert PF. Both conserved region 1 (CR1) and CR2 of the human papillomavirus type 16 E7 oncogene are required for induction of epidermal hyperplasia and tumor formation in transgenic mice. *J Virol* 1997; **71:** 5905-5914.

7 Porreco R *et al.* Gynecological malignancies in immunosuppressed organ homograft recipients. *Obstet Gynecol* 1975; **45:** 359-364.

8 Johnson JC *et al.* High frequency of latent and clinical human papillomavirus cervical infections in immunocomprised human immunodeficiency virus-infected women. *Obstet Gynecol* 1992; **79:** 321-327.

9 Feltkamp MC *et al.* Vaccination with cytotoxic T lymphocyte epitope containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993; **23:** 2242-2249.

10 Feltkamp MC *et al.* (1995) Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. *Eur J Immunol* 1995; **25:** 2638-2642.

11 Jochmus I *et al.* Specificity of human cytotoxic T lymphocytes induced by a human papillomavirus type 16 E7-derived peptide. *J Gen Virol* 1997; **78:** 1689-1695.

12 De Bruijn ML *et al.* Immunization with Human Papillomavirus Type 16 (HPV16) Oncoprotein-loaded dendritic cells as well as prrotein in adjuvant induces MHC Class I-restricetd protection to HPV16-induced tumor cells. *Cancer Res* 1998; **58:** 724-731.

13 Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. *Microbiology Reviews* 1994; **58**: 491-562.

14 Liljeström P, Lusa S, Huylebroeck D, Garoff H. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol* 1991; **65:** 4107-4113.

15 Liljeström P, Garoff H. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnol* 1991; **9:** 1356-1361.

16 Berglund P *et al.* Semliki Forest virus expression system: Production of conditionally infectious recombinant particles. *Biotechnol* 1993; **11:** 916-920.

17 Sedman SA *et al.* The full-lenght E6 protein of Human Papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *J Virol* 1991; **65:** 4860-4866.

18 Greenfield I, Nickerson J, Penman S, Stanley M. Human papillomavirus 16 E7 protein is associated with the nuclear matrix. *Proc Natl Acad SciUSA* 1991; **88:** 11217-11221.

19 Nindl I *et al.* The E7 protein of human papillomavirus (HPV) type 16 expressed by recombinant vaccinia virus can be used for detection of antibodies in sera from cervical cancer patients. *J Virol Methods* 1996; **62:** 81-85.

20 Braspenning J *et al.* A general purification protocol for E7 proteins from Ahigh- and low-risk@ Human Papillomavirus types expressed in the yeast *Schizosaccharamyces pombe*. *Protein Expression Purif* 1997; **10:** 192-201.

21 Grossman SR, Mora R, Laimins LA. Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. *J Virol* 1989; **63:** 366-374.

22 Daniels PR, Sanders CM, Maitland NY. Characterization of the interactions of human papillomavirus type 16 E6 with p53 and E6-associated protein in insect and human cells. *J Gen Virol* 1998; **79:** 489-499.

23 Toes REM *et al.* Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc Natl Acad Sci USA* 1996; **93:** 7855-7860.

24 Boursnell MEG *et al.* Construction and characterization of a recombinant vaccinia expressing Human Papillomavirus proteins for immunotherapy of cervical cancer. *Vaccine*1996; **14:** 1485-1494.

25 Lin KY *et al.* Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; **56:** 21-26.

26 Chen L *et al.* Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papillomavirus type 16. *J Immunol* 1992; **148:** 2617-2621.

27 Borysiewicz LK *et al.* A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 1996; **347:** 1523-1527.

28 Glasgow GM, McGee MM, Sheahan BJ, Atkins GJ. Death mechanisms in cultured cells infected by Semliki Forest virus. *J Gen Virol* 1997; **78:** 1559-1563.

29 Berglund P, Fleeton MN, Smerdou C, Liljeström P. Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice. *Vaccine* 1999; **17:** 497-507.

30 Rock KL. A new foreign policy: MHC class I molecules monitor the outside world. *Immunol Today* 1996; **17:** 131-137.

31 Bennett SRM. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998; **393:** 478-480.

32 Lanzavecchia A. Immunology. Licence to kill *Nature* 1998; **393:** 413-414.

33 Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell *Nature* 1998; **393:** 474-478.

34 Schoenberger SP *et al.* T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions *Nature* 1998; **393:** 480-483.

35 Zhou X *et al.* Generation of cytotoxic and humoral immune responses by nonreplicative recombinant Semliki Forest virus. *Proc Natl Acad Sci USA* 1995; **92:** 3009-3013.

36 Berglund P *et al.* Outcome of immunization of Cynomolgus monkeys with recombinant Semliki Forest virus encoding Human Immunodeficiency virus type I envelope protein and challenge with a high dose of SHIV-4 virus. *AIDS Res Hum Retroviruses* 1997; **13:** 1487-1495.

37 Smerdou C, Liljeström P. Two-helper RNA system for production of recombinant Semliki forest virus particles. *J Virol* 1999; **73:** 1092-1098.

38 Smits PHM, Smits HL, Jebbink MF, Ter Schegget J. The short arm of chromosome 11 likely is involved in the regulation of the human papillomavirus type 16 early enhancer-promoter and in the suppression of the transforming activity of the viral DNA. *Virol* 1990; **176:** 158-165.

Titles and legends to figures

Figure 1 Western blot analysis of SFV-E6E7 transfected BHK cell extracts. BHK cells were infected with SFV-E6E7 particles or SFV-LacZ particles. After overnight incubation, the cellular proteins were extracted and analyzed by SDS-PAGE and immunoblotting. E6 was detected using a polyclonal rabbit-anti-HPV16 E6 antibody (section A), E7 was detected using a monoclonal mouse-anti-HPV16 E7 antibody (section B). Lanes 1: BHK21 cells not infected; lanes 2: BHK21 cells infected with SFV-E6E7 particles; lanes 3: BHK21 cells infected with SFV-LacZ particles; M: protein marker

Figure 2 Intracellular localization of E7 in SFV-E6E7 infected BHK cells. BHK21 cells were infected with SFV-E6E7. After overnight incubation, the cells were stained using anti-HPV16 E6 or anti-HPV16 E7 antibodies. A: Immunofluorescent staining of SFV-E6E7 infected cells with anti-HPV16 E6, B: Immunofluorescent staining of SFV-E6E7 infected cells with anti-HPV16 E7. Magnification 40 x.

Figure 3 CTL activity induced upon immunization with SFV-E6E7 particles, as determined after an 11 and 18 day in vitro restimulation. Mice were immunized s.c. and boosted twice (s.c. and i.p) with purified 10^6 SFV-E6E7 (n=4, open and closed squares and diamonds), SFV-LacZ particles (triangles) or PBS (crosses), as a control. CTL activity was determined one week after the last booster immunization. After 11 days (panel A) and 18 days (panel B) in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 4 Recognition of and lysis HPV16-transformed C3 cells as well as 13-2 cells expressing the H-2D^b-binding HPV16 CTL epitope by CTLs induced upon immunization with SFV-E6E7 particles. Mice were immunized s.c. and boosted twice (s.c. and i.p) with purified 10^6 SFV-E6E7 (n=2, squares and diamonds), SFV-LacZ particles (open triangles) or PBS (crosses), as a control. After 11 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells (panel A) and C3 cells (panel B) in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 5 Recognition of E7 49-57-loaded syngeneic EL4 cells by CTLs induced upon immunization with SFV-E6E7 particles. Mice were immunized s.c. and boosted twice (s.c. and i.p) with purified 10^6 SFV-E6E7 (n=2, squares and diamonds), SFV-LacZ particles (triangles) or PBS (crosses), as a control. After 18 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against E7 49-57-loaded EL4 cells (panel A) and unloaded EL4 cells (panel B) in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 6 CTL activity in mice immunized with various doses of SFV-E6E7 particles. In two separate experiments, mice were immunized s.c. and boosted twice (s.c. and i.p) with purified 10^4 (solid triangles), 10^5 (solid diamonds) or 10^6 (solid squares) SFV-E6E7 particles, 10^6 SFV-LacZ particles (triangles) or PBS (crosses), as a control. CTL activity was determined one week after the last booster immunization. After 18 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios of two individual experiments. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 7 Growth of TC-1 tumor cells in SFV-E6E7 immunized mice. Mice were immunized s.c. and boosted twice (s.c. and i.p.) with PBS (Panel A; n=10), 5x10^6 SFV-LacZ particles (Panel B; n=10), 10^6 SFV-E6E7 particles (Panel C; n=10) or 5x10^6 SFV-E6E7 particles (Panel D; n=5). Tumor growth was monitored twice weekly. Each line represents the tumorvolume of a separate mouse.

Figure 8 Growth of TC-1 tumor cells in SFV-E6E7 immunized mice. Mice were immunized s.c. and boosted twice (s.c. and i.p.) with PBS (n=10; open circles), $5x10^6$ SFV-LacZ particles (n=10; open squares), 10^6 SFV-E6E7 particles (n=10; solid diamonds) or $5x10^6$ SFV-E6E7 particles (n=5; solid squares). Tumor growth was monitored twice weekly. Shown are the percentages of mice with non-palpable tumors.

Figure 9 Cloning strategy for the construction of SFV-enhE6,7
Out of the pSFV3-E6E7 the E6 sequence was modified with an NcoI site at the 5' end and an EcoRI site at the 3' end. The E7 sequence was modified with an EcoRI site at the 5' end and a BamHI site at the 3' end by PCR. The 5' end of the capsid gene of SFV coding for the first 34 amino acid residues has been shown to contain a translational enhancer. This enhancer was cloned in a pSFV-helper-S1 construct by Smerdou and Liljestrom (J.Virol. 73, 1092-1098, 1999). In addition they inserted the sequence of foot-and-mouse disease virus (FMDV) 2A autoprotease (17 amino acids) in frame between the translational enhancer and p62 (SFV envelope protein) in order to provide cleavage between the proteins. We synthesized the sequence containing the translational enhancer and the FMDV A2 autoprotease from pSFV-helper-S1, and by PCR BamHI and NcoI restriction sites were generated at the 5' and 3' end, respectively. The enh-FMDV A2 protease-, E6- and E7 fragments were cloned into the BamHI site of pSFV3, producing pSFV3-eE6,7.

In the original plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. In pSFV3-eE6,7 one base pair is inserted between E6 and E7 and the stop codon TAA of E6 is changed in GAA. Thus, in pSFV3-eE6,7 the sequence encoding E6 and E7 is in frame, expressing a fusion product of E6 and E7.

Figure 10 Western blot analysis of recSFV transfected BHK cell extracts. BHK cells were infected with SFV-E6E7, SFV-eE6E7 or SFV-eE6,7 particles. After overnight incubation, the cellular proteins were extracted and analysed by SDS-PAGE and immunoblotting. E6 was detected using a polyclonal rabbit-anti-HPV16 E6 antibody (lanes1-3); E7 was detected using a monoclonal mouse-anti-HPV16 E7 antibody (lanes 5-8). Lanes 1 and 5: BHK21 cells infected with SFV-E6E7 particles; Lanes 2 and 6: BHK21 cells infected with SFV-eE6E7 particles; Lane 3 and 7: BHK21 cells infected with SFV-eE6,7 particles; Lane 4: protein marker (M); Lane 8: recombinant (E.coli) produced E7 protein.

Figure 11 Analysis of E6 and E7 expression by pulse labelling. BHK cells were infected with SFV-E6E7, SFV-eE6,E7 or SFV-eE6,7 particles. After 6 hr, the cells were cultured for an additional 30-min with methionine- and cysteine-free medium followed by a 1-h labelling-period with ^{35}S-methionine/cysteine. After one hr the cells were washed and harvested or cultured for an additional 6, 16 or 40 h before harvesting. Cell lysates were analysed by SDS/PAGE and autoradiography. Lane 1: BHK21 cells not infected, analysed after a 6 h chase; Lane 2:BHK21 cells infected with SFV-E6E7, analysed after a 6 h chase; Lanes 3-5: BHK cells infected with SFV-eE6E7, analysed directly or after 6 h or 16 h chase, respectively; Lanes 6-9: BHK21 cells infected with SFV-eE6,7, analysed directly or after 6 h, 16 h or 40 h chase, respectively.

Figure 12 CTL activity induced upon immunisation with SFV-E6E7 particles, as determined after a 7 and 14 day in vitro restimulation. Mice were immunised s.c. and boosted twice (s.c. and i.p) with purified 10^6 SFV-eE6,7 (n=2, closed squares), 10^5 SFV-eE6,7 (n=2, open squares), 10^6 SFV-E6E7 (n=2, closed circles), 10^5 SFV-E6E7 particles (n=2, open circles) or with 10^6 SFV-LacZ (n=2, closed triangles) or PBS (n=2, open triangles) as controls. CTL activity was determined one week after the last booster immunisation. After 7 days (panel A) and 14 days (panel B) in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 13 CTL activity induced upon immunisation with 1, 2.5 and 5x10^6 SFV-eE67 particles. Mice were immunised s.c. and boosted twice (s.c. and i.p) with purified 5x10^6 SFV-eE6,7 (closed squares), 2.5x10^6 SFV-eE6,7 (open circles), 10^6 SFVeE6,7 (open squares) or with 5x10^6 SFV-LacZ (closed triangles) or PBS (open triangles) as controls. CTL activity was determined 18 days (Panel A) or 8 weeks (Panel B) after the last booster immunisation. After 7 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 14 Induction of long-term CTL activity requires a single booster immunisation. Mice received a single s.c. injection of 2.5x10^6 SFV-eE6,7 particles (Panel A) or two s.c. injections of 2.5x10^6 SFV-eE6,7 particles (Panel B). CTL activity was determined 10 days (squares), 1 month (circles) or three months (triangles) after the (last) injection of particles. After 7 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. The standard errors of the means of the triplicate determinations were always <10% of the value of the mean.

Figure 15 CTL activity and precursor CTL frequency in mice immunised with SFV-eE6,7 particles via different routes. Mice were immunised and boosted twice with purified 10^6 SFV-eE6,7 particles s.c. (n=2), i.p. (n=3) or i.v. (n=3). Spleen cells were isolated one week after the last booster immunisation. For CTL activity, spleen cells were restimulated for 7 days in vitro. The resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. In the upper three panels the levels of cytolysis at different effector to target ratios are given. For tetramer staining, spleen cells were stained directly after isolation with anti-CD8-FITC antibody and an HPV16-E7 specific MHC class I tetramer (PE-labelled). In the lower three panels the percentages of CD8$^+$/ tetramer$^+$ T cells are given of the individual mice. The filled, grey and open bars in the lower panels correspond to the levels of CTL activity of the filled, grey and open symbols in the upper panels.

Figure 16 Protection from growth of TC-1 tumour cells in SFV-eE6,7 immunised mice. Mice were immunised s.c. and boosted twice (s.c. and i.p.) with PBS (n=10; open triangles), 10^6 SFV-LacZ particles (n=10; open squares) or 10^6 SFV-E6,7 particles (n=10; solid squares). Tumour growth was monitored twice weekly. Shown are the percentages of mice with non-palpable tumours in time.

Figure 17 Therapeutic treatment of TC-1 tumours by SFV-eE6,7 immunisation. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1. At several time points after tumour inoculation mice were injected s.c with $5x10^6$ SFV-eE6,7 particles (lower three panels). One group of mice (n=7) was immunised on days 2, 7 and 14 after tumour inoculation, a second group (n=14) was immunised on days 7, 14 and 21 after inoculation and the last group (n=6) was immunised at days 14, 21 and 28 after inoculation. In addition two control groups were included that were injected with either PBS (n=9) or $5x10^6$ SFV-LacZ particles (n=5) on days 2, 7 and 14 after tumour inoculation. Tumour growth was monitored twice weekly. Each line represents the tumour volume of a separate mouse. Given are the combined results of two experiments.

Figure 18 Therapeutic treatment of TC-1 tumours by SFV-eE6,7 immunisation. Combined results of figure 17 showing the percentages of tumour-free mice in time. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1. At several time points after tumour inoculation mice were injected with SFV-eE6,7 particles as described in the legend to figure 17. Shown are the percentages of tumour-free mice after injection of SFV-eE6,7 on days 2, 7 and 14 after tumour inoculation (n=7, filled squares), after injection of SFV-eE6,7 on days 7, 14 and 21 (n=14, open diamonds) or after injection with PBS (n=9, open circles) or $5x10^6$ SFV-LacZ particles (n=5, open triangles) on days 2, 7 and 14 after tumour inoculation. Tumour growth was monitored twice weekly.

Figure 19 Nucleotide sequence of construct enhE6,7

Figure 20 Tumour rechallenge of mice surviving a first tumour challenge. The left panel depicts the percentages of tumour-free mice in time of one of the experiments as shown and described in figure 17. 13 weeks after the first tumour inoculation mice were rechallenged with 2x10^4 TC-1 without additional immunization. The right panel shows the percentages of tumour-free mice upon tumour rechallenge of mice originally immunized with SFV-eE6,7 on days 2, 7 and 14 (n=7, squares) or with SFV-eE6,7 on days 7, 14 and 21 (n=4, diamonds). Since all control mice had developed a tumour upon the first tumour challenge, 4 control mice were included in the rechallenge experiment (circles). Tumour growth was monitored twice weekly

Figure 21 Therapeutic treatment of TC-1 tumours by intravenous immunization with SFV-eE6,7 particles. Mice were inoculated s.c. in the neck with 2x10^4 TC-1. At several time points after tumour inoculation mice were injected i.v. with 5x10^6 SFV-eE6,7 particles or PBS. Mice were immunised on days 7, 14 and 21 (n=7; middle panel) after tumour inoculation or on days 14, 21 and 28 (n=7, right panel) after tumour inoculation inoculation. In addition one buffer (PBS) control group was included (n=5, left panel). Tumour growth was monitored twice weekly. Each line represents the tumour volume of a separate mouse.

Figure 22 Therapeutic treatment of TC-1 tumours by intravenous immunization with decreasing amounts of SFV-eE6,7 particles. Mice were inoculated s.c. in the neck with 2x10^4 TC-1. At several time points after tumour inoculation mice were injected i.v. with 5x10^6 SFV-eE6,7 particles (n=7; lower left panel), 5 x 10^5 SFV-eE6,7 particles (n=7; lower middle panel), 5x10^4 SFV-eE6,7 particles (n=7; lower right panel) or with PBS (n=5; upper panel) on days 7, 14 and 21 after tumour inoculation. Tumour growth was monitored twice weekly. Each line represents the tumour volume of a separate mouse.

Claims

1. An alphavirus vector system comprising nucleic acid derived from a human papilloma virus (HPV).
2. A vector system according to claim 1 wherein said nucleic acid encodes at least one antigenic polypeptide fragment of said HPV
3. A vector system according to claim 2 wherein said fragment is derived from protein E6 and/or protein E7 of HPV.
4. A vector system according to claim 3 wherein said fragment comprises one antigenic polypeptide fragment of E6 and one antigenic polypeptide fragment of E7.
5. A vector system according to claim 3 or 4 wherein said fragment has at least partially been deprived of the capacity to bind to pRb and/or P53 protein.
6. A vector system according to anyone of claims 1 to 5 further comprising an translational enhancer element.
7. A vector system according to anyone of claims 1 to 6 further comprising a nucleic acid encoding an auto-protease.
8. A vector system according to claim 7 wherein said auto-protease is derived from foot-and-mouth-disease virus.
9. A vector system according to anyone of claims 1 to 8 wherein an alphavirus structural and a non-structural protein are expressed from at least two independent nucleic acid molecules.
10. A vector system according to anyone of claims 1 to 9 wherein said alphavirus comprises Semliki Forest Virus.
11. A vector system according to anyone of claims 1 to 10 wherein said HPV comprises HPV16 and/or HPV18.
12. A vector system according to anyone of claims 1 to 11 wherein said nucleic acid further encodes a cytokine gene or functional fragment thereof,
13. A vector system according to claim 12 wherein said cytokine comprises GM-CSF or IL12.
14. A cell comprising a vector system according to anyone of claims 1 to 13.

15. Use of a vector system according to anyone of claims 1 to 13 or a cell according to claim 14 for the preparation of a medicament.
16. Use according to claim 15 for the preparation of a medicament for the treatment or prevention of cervical cancer.
17. Use according to claim 15 or 16 wherein said medicament is a vaccine.
18. A medicament comprising a vector system according to anyone of claims 1 to 13 or a cell according to claim 14.
19. A medicament according to claim 18 for the treatment or prevention of cervical cancer.
20. A method for the treatment or prevention of cervical cancer comprising providing an individual with a medicament according to claim 19.
21. A method according to claim 21 further comprising providing said individual with an additional cytokine or fragment thereof.

FIGURE 1

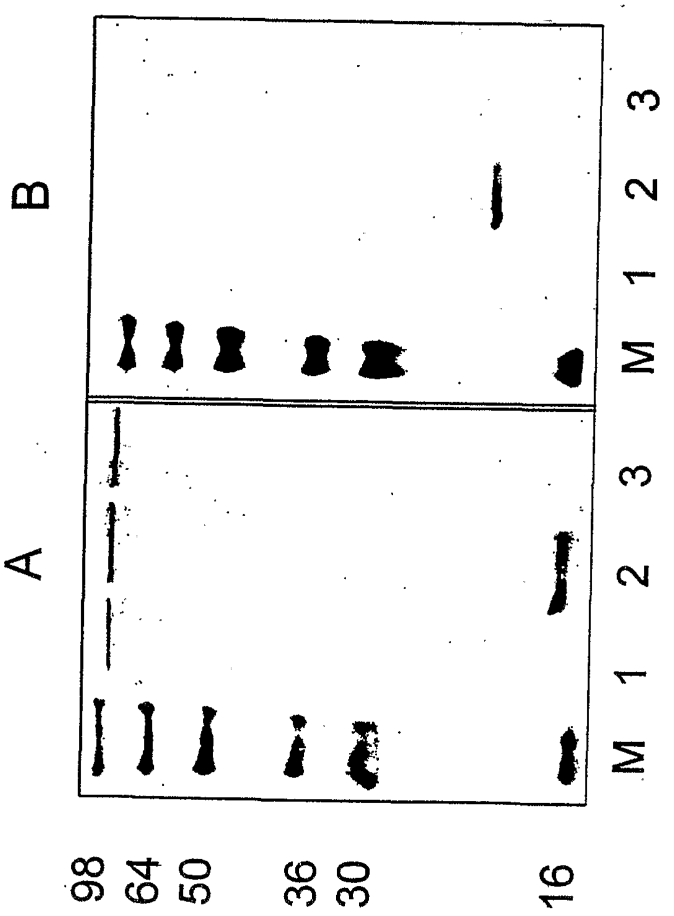


FIGURE 2

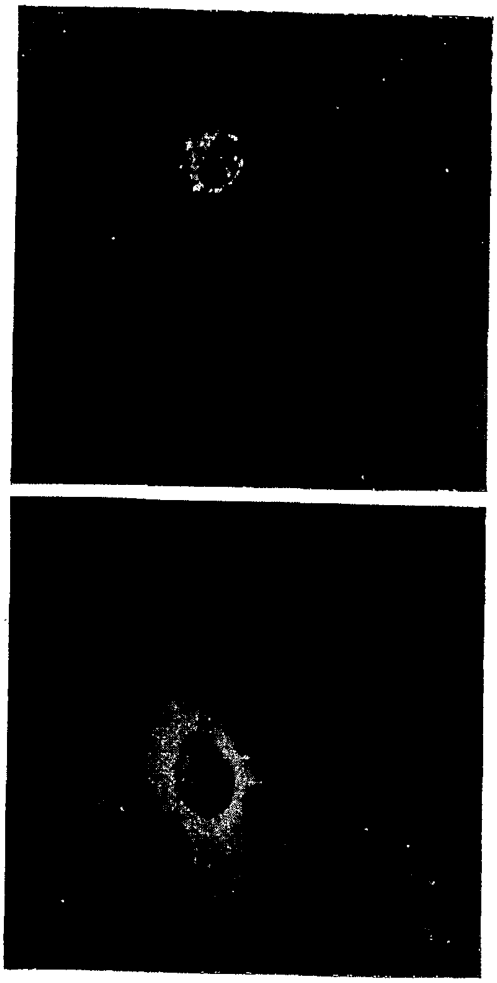


FIGURE 4

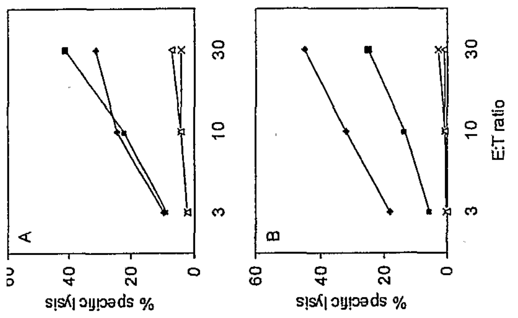


FIGURE 6

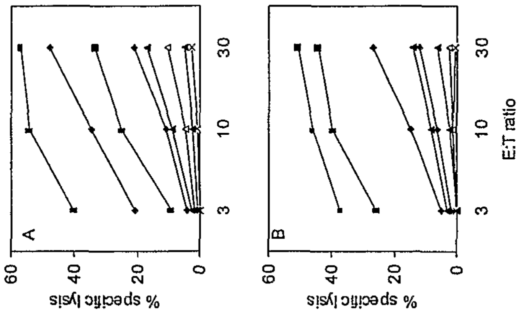


FIGURE 3

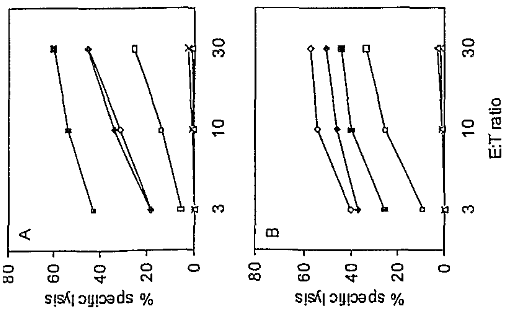


FIGURE 5

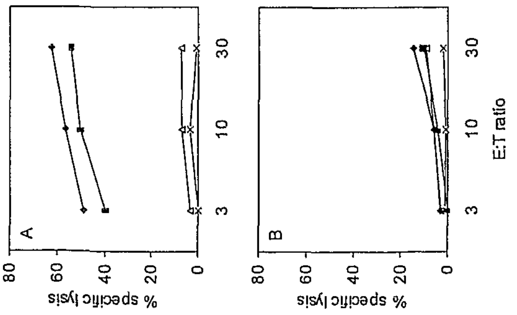


FIGURE 7

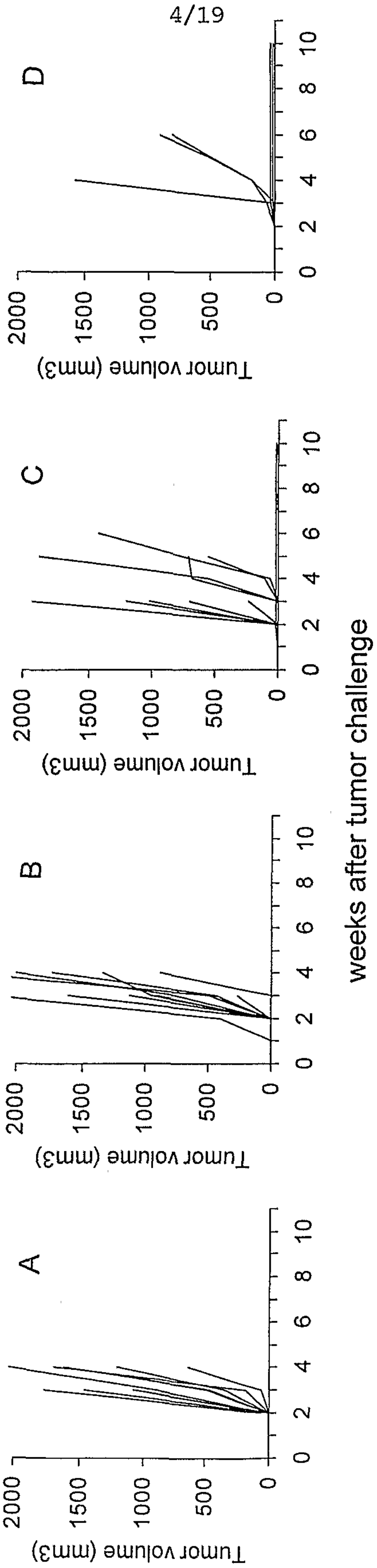

A
B
C
D
Tumor volume (mm3)
2000
1500
1000
500
0
0
2
4
6
8
10
weeks after tumor challenge

FIGURE 8

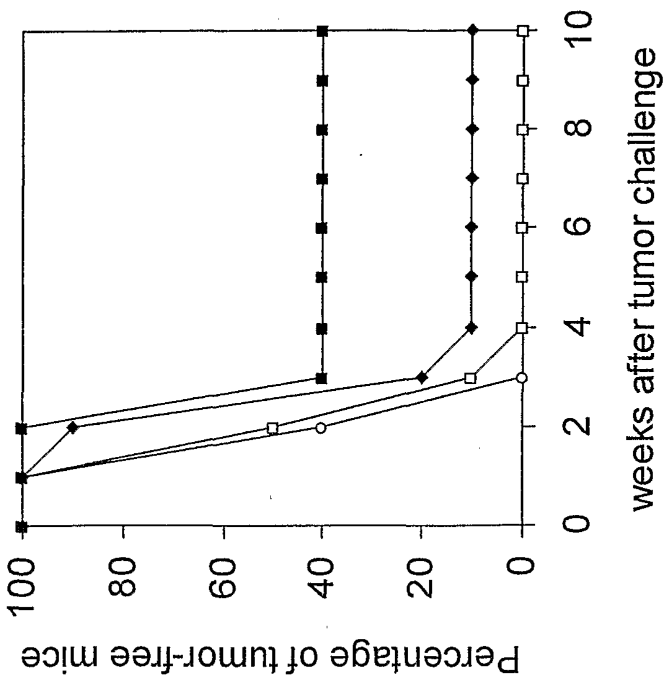

Percentage of tumor-free mice
100
80
60
40
20
0
weeks after tumor challenge
0
2
4
6
8
10

FIGURE 9

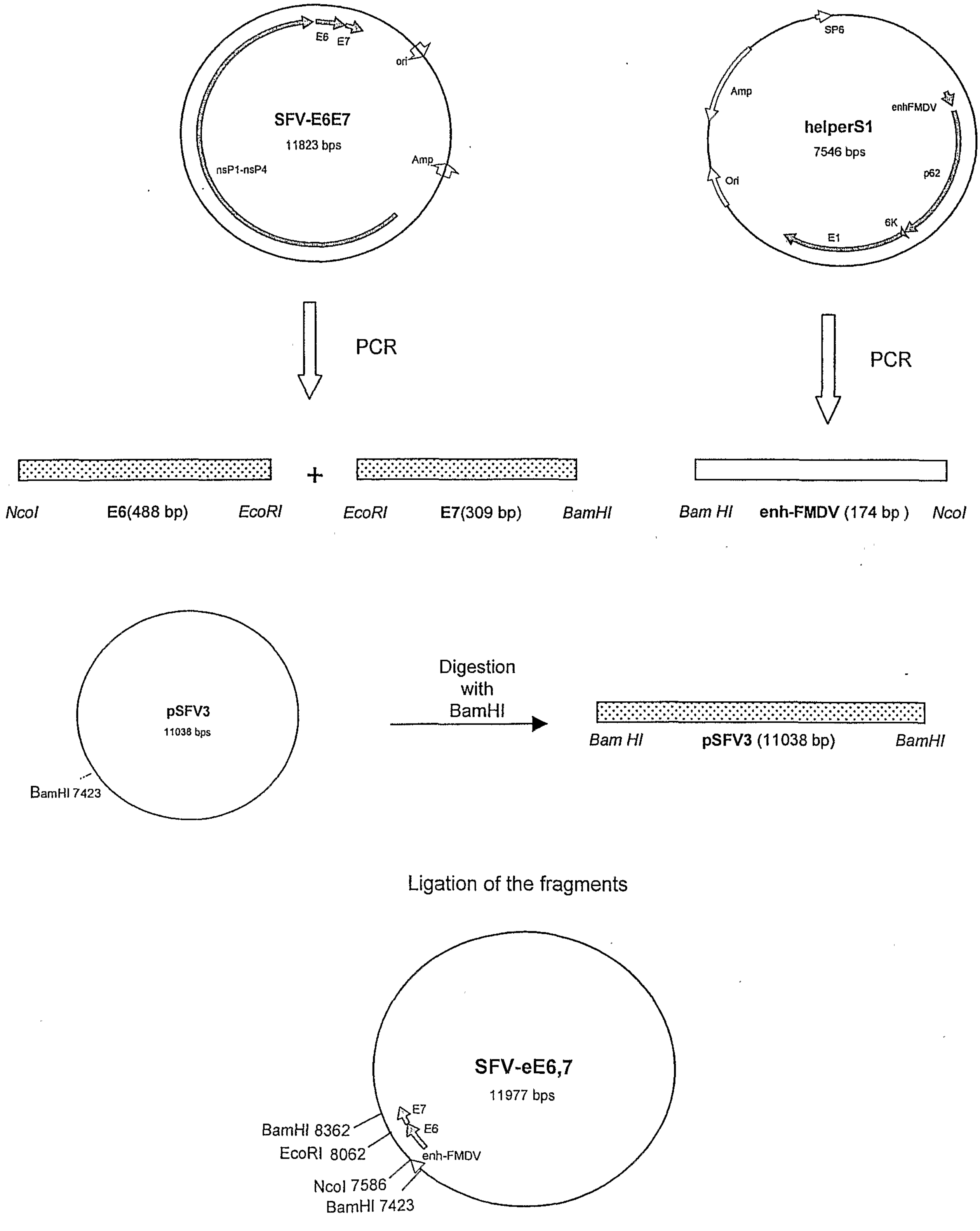

SFV-E6E7
11823 bps
E6
E7
ori
Amp
nsP1-nsP4
helperS1
7546 bps
SP6
Amp
enhFMDV
p62
Ori
6K
E1
PCR
PCR
NcoI
E6(488 bp)
EcoRI
+
EcoRI
E7(309 bp)
BamHI
Bam HI
enh-FMDV (174 bp)
NcoI
pSFV3
11038 bps
BamHI 7423
Digestion with BamHI
Bam HI
pSFV3 (11038 bp)
BamHI
Ligation of the fragments
SFV-eE6,7
11977 bps
E7
E6
enh-FMDV
BamHI 8362
EcoRI 8062
NcoI 7586
BamHI 7423

FIGURE 10

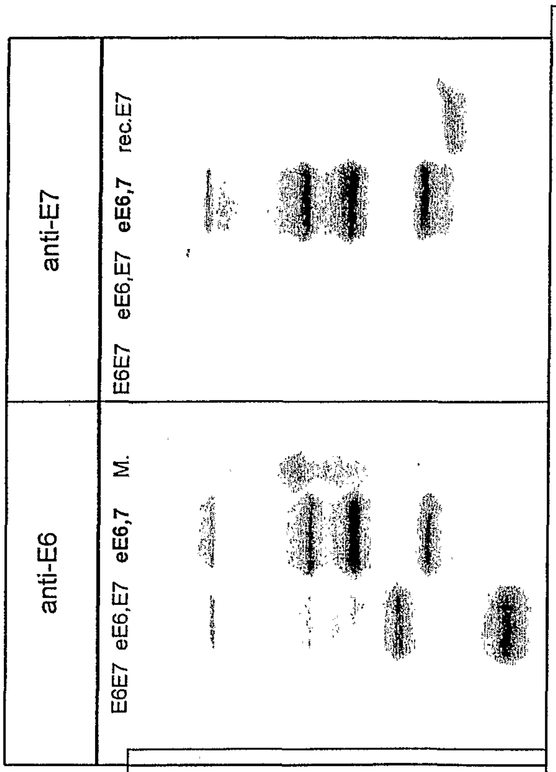


FIGURE 11

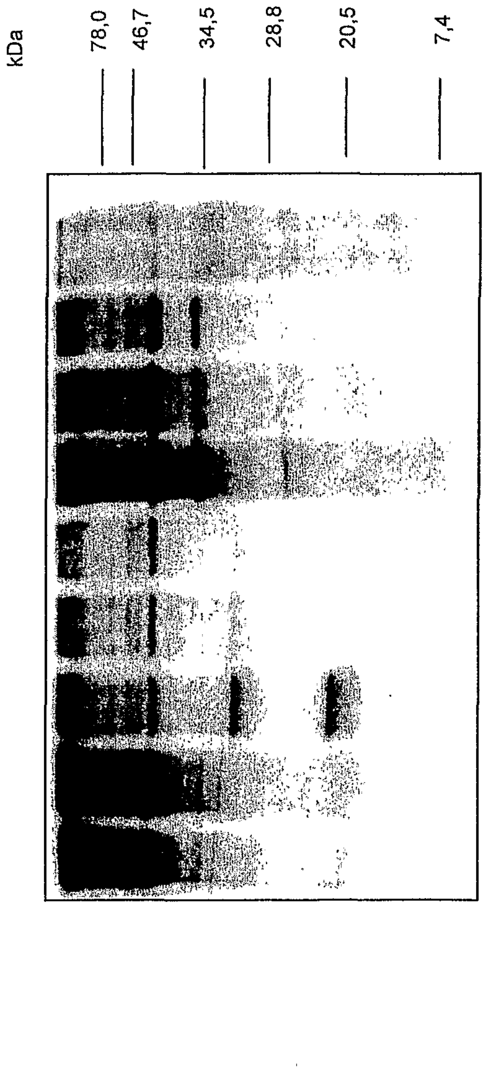


FIGURE 12

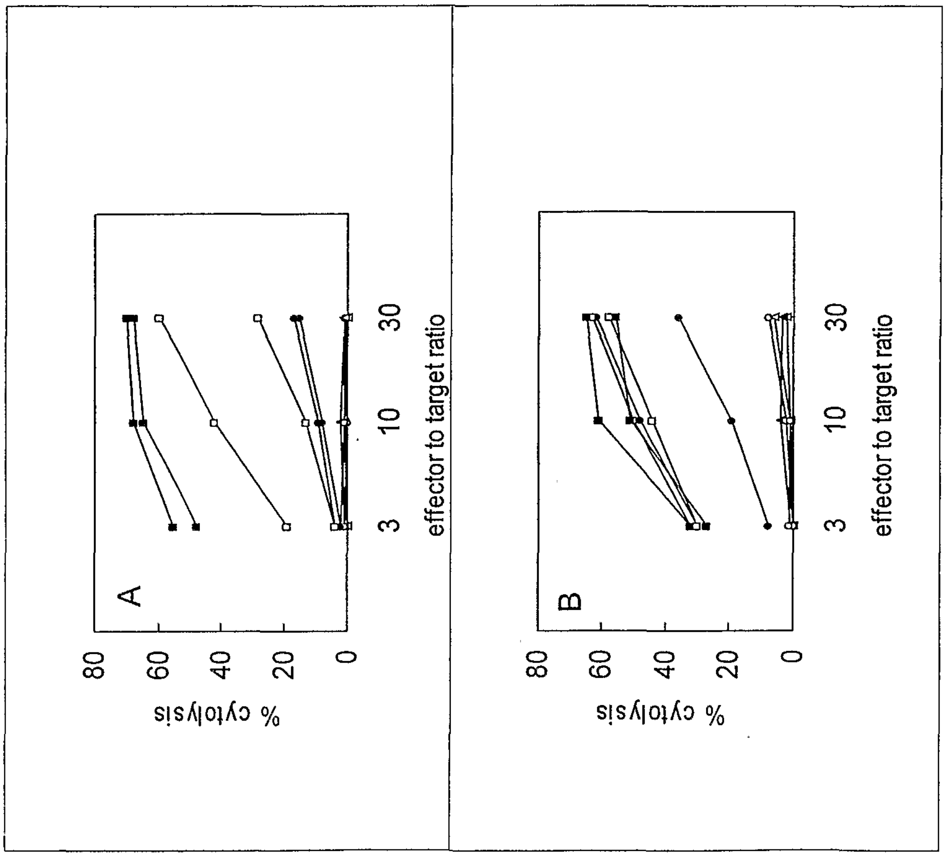

A
B
% cytolysis
% cytolysis
0
20
40
60
80
effector to target ratio
effector to target ratio
3
10
30

FIGURE 13

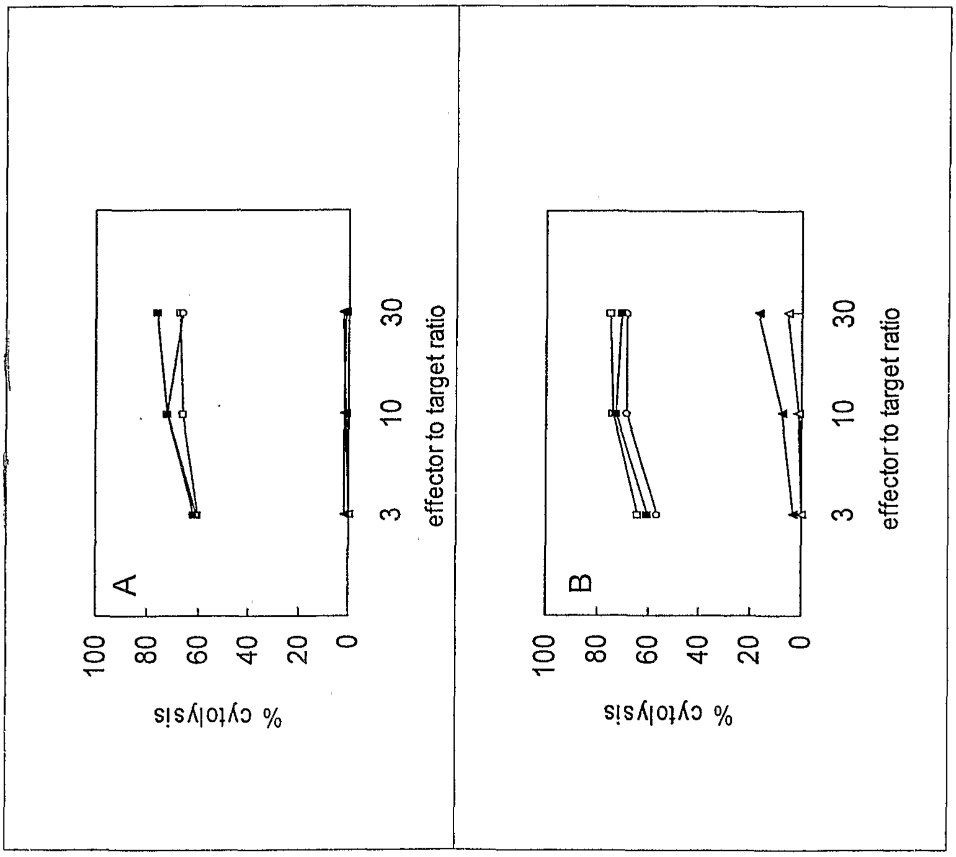

A
% cytolysis
100
80
60
40
20
0
3
10
30
effector to target ratio
B
% cytolysis
100
80
60
40
20
0
3
10
30
effector to target ratio

FIGURE 14

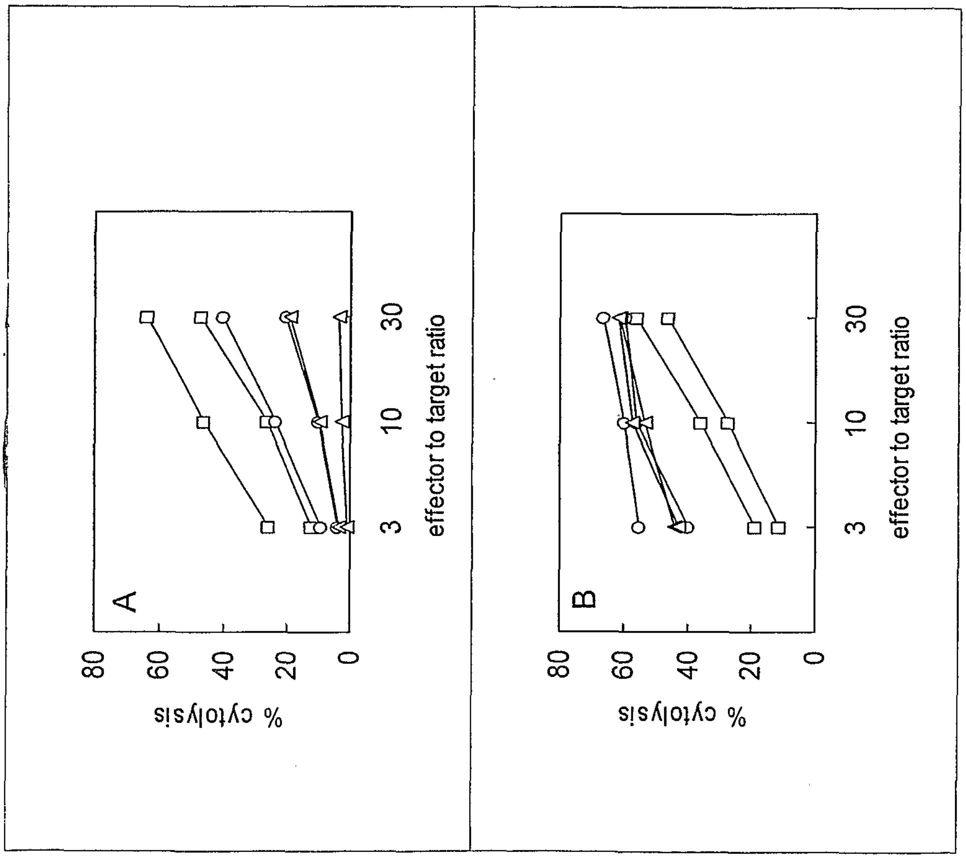


FIGURE 15

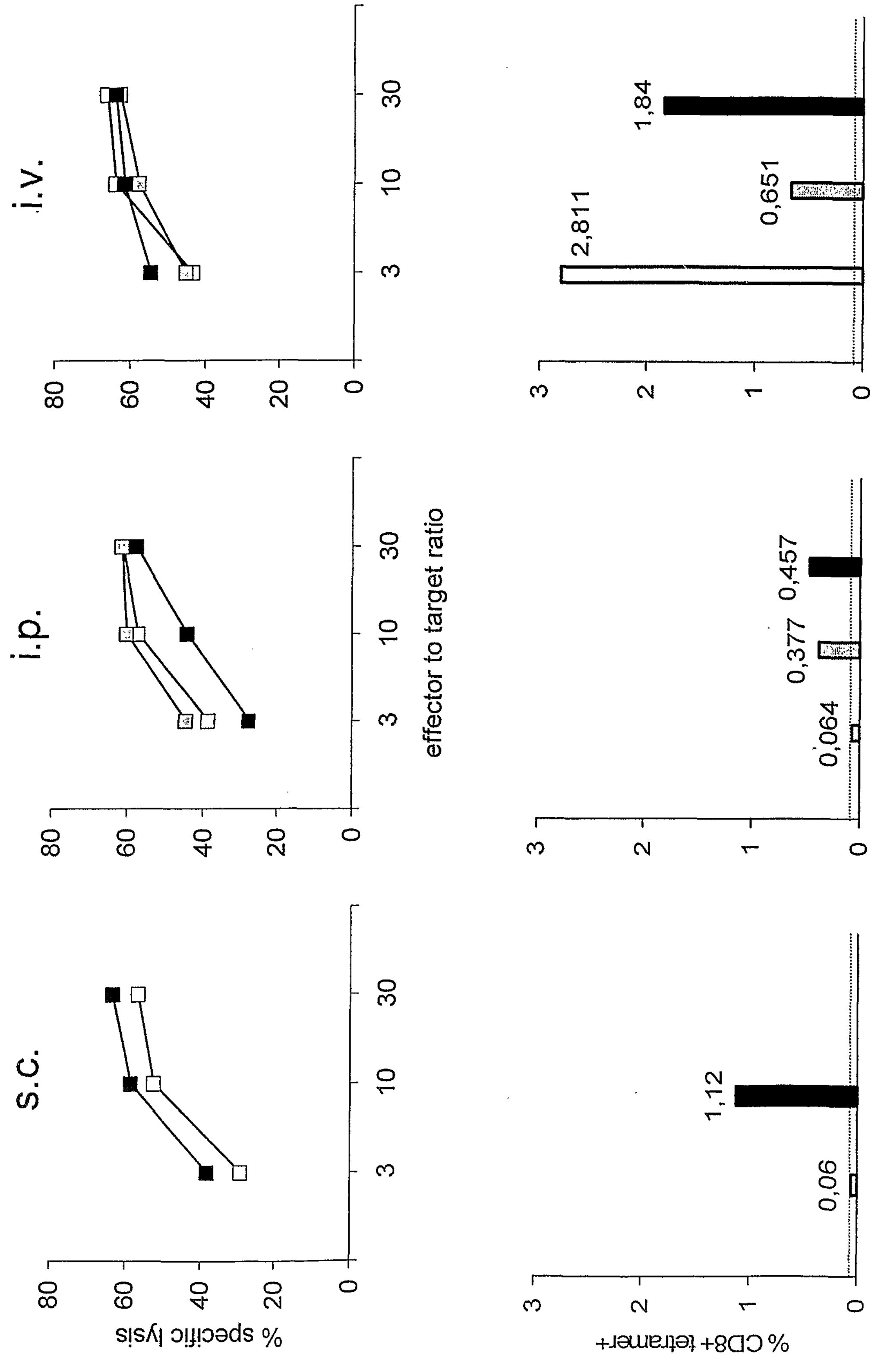

s.c.
i.p.
i.v.
% specific lysis
0
20
40
60
80
3
10
30
effector to target ratio
% CD8+ tetramer+
0
1
2
3
0,06
1,12
0,064
0,377
0,457
2,811
0,651
1,84

FIGURE 16

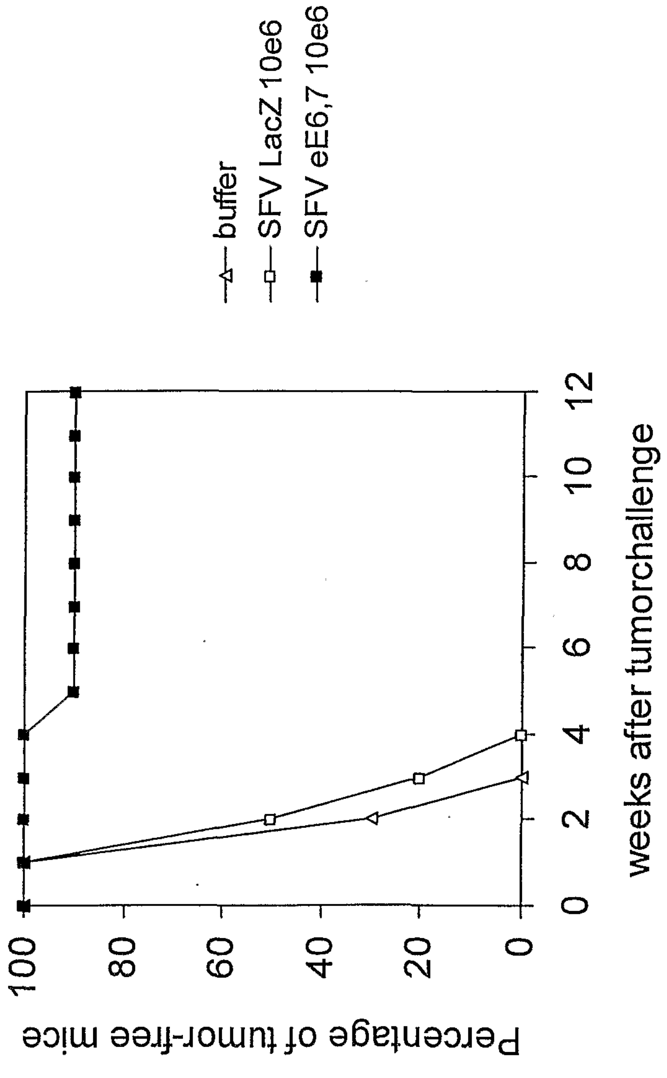

buffer
SFV LacZ 10e6
SFV eE6,7 10e6
Percentage of tumor-free mice
100
80
60
40
20
0
weeks after tumorchallenge
0
2
4
6
8
10
12

FIGURE 17

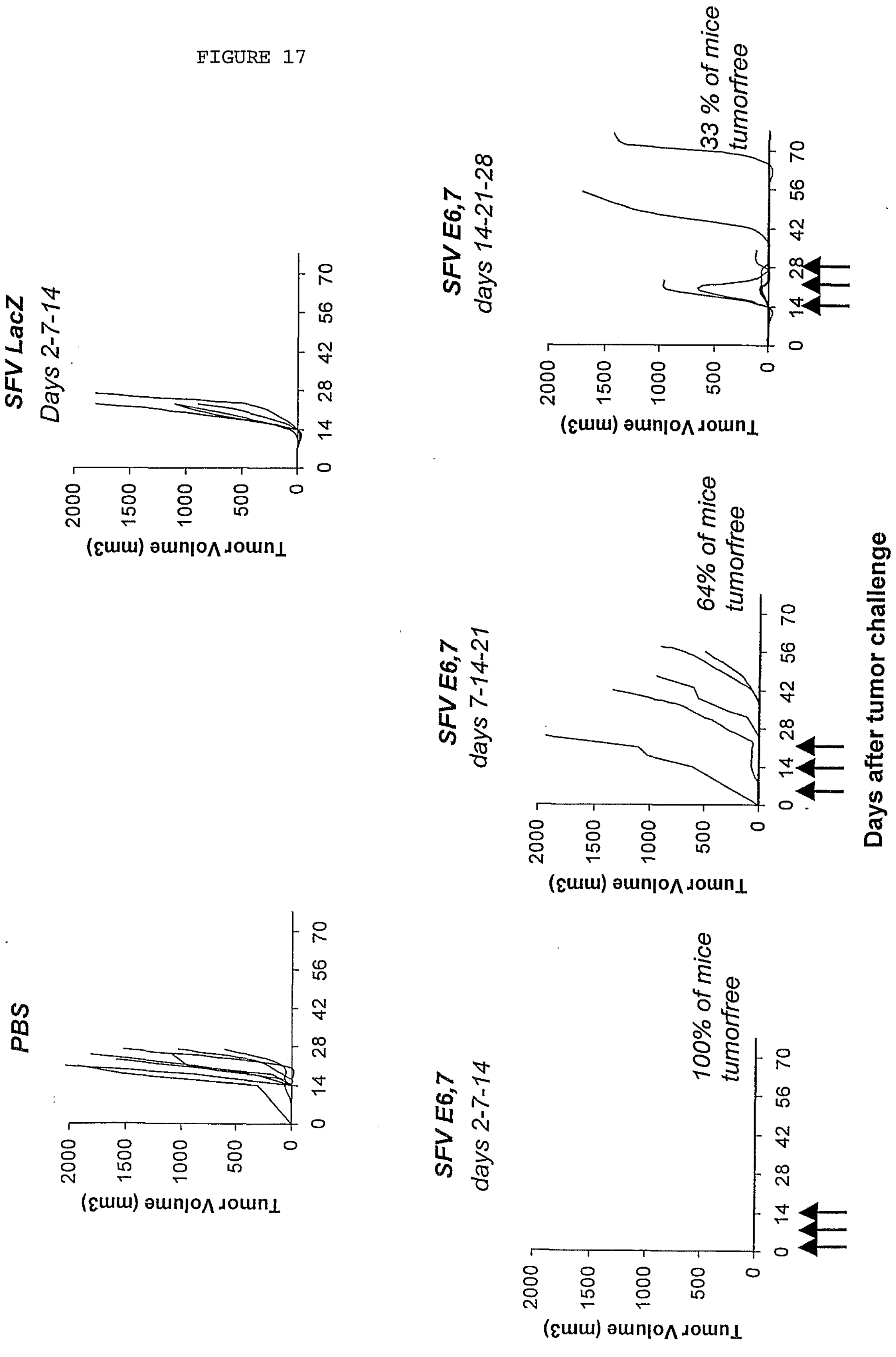

PBS
SFV LacZ
Days 2-7-14
SFV E6,7
days 2-7-14
100% of mice tumorfree
SFV E6,7
days 7-14-21
64% of mice tumorfree
SFV E6,7
days 14-21-28
33 % of mice tumorfree
Tumor Volume (mm3)
2000
1500
1000
500
0
0 14 28 42 56 70
Days after tumor challenge

FIGURE 18

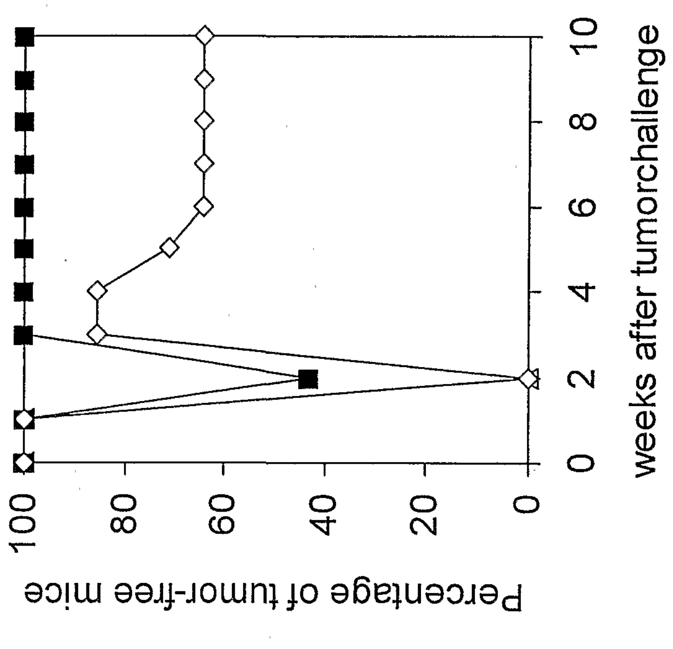

Percentage of tumor-free mice
100
80
60
40
20
0
weeks after tumorchallenge
0
2
4
6
8
10

FIGURE 19

enhE6,7 sequence

GATCCAGCACCATGAATTACATCCCTACGCAAACGTTTTACGGCCGCCGGTGGCG
CCCGCGCCCGGCGGCCCGTCCTTGGCCGTTGCAGGCCACTCCGGTGGCTCCCGT
CGTCAATTTTGACCTTCTTAAGCTTGCGGGAGACGTCGAGTCCAACCCTGGGCCC
ATGGACCAAAAGAGAACTGCAATGTTTCAGGACCCACAGGAGCGACCCAGAAAG
TTACCACAGTTATGCACAGAGCTGCAAACAACTATACATGATATAATATTAGAAT
GTGTGTACTGCAAGCAACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCG
GGATTTATGCATAGTATATAGAGATGGGAATCCATATGCTGTATGTGATAAATGT
TTAAAGTTTTATTCTAAAATTAGTGAGTATAGACATTATTGTTATAGTTTGTATGG
AACAACATTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTAGGTGT
ATTAACTGTCAAAAGCCACTGTGTCCTGAAGAAAAGCAAAGACATCTGGACAAAA
AGCAAAGATTCCATAATATAAGGGGTCGGTGGACCGGTCGATGTATGTCTTGTTG
CAGATCATCAAGAACACGTAGAGAAACCCAGCTGGAATTCATGGAGATACACCTA
CATTGCATGAATATATGTTAGATTTGCAACCAGAGACAACTGATCTCTACTGTTAT
GAGCAATTAAATGACAGCTCAGAGGAGGAGGATGAAATAGATGGTCCAGCTGGA
CAAGCAGAACCGGACAGAGCCCATTACAATATTGTAACCTTTTGTTGCAAGTGTG
ACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTCGTACTTTGGA
AGACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCA
TAACG

FIGURE 20

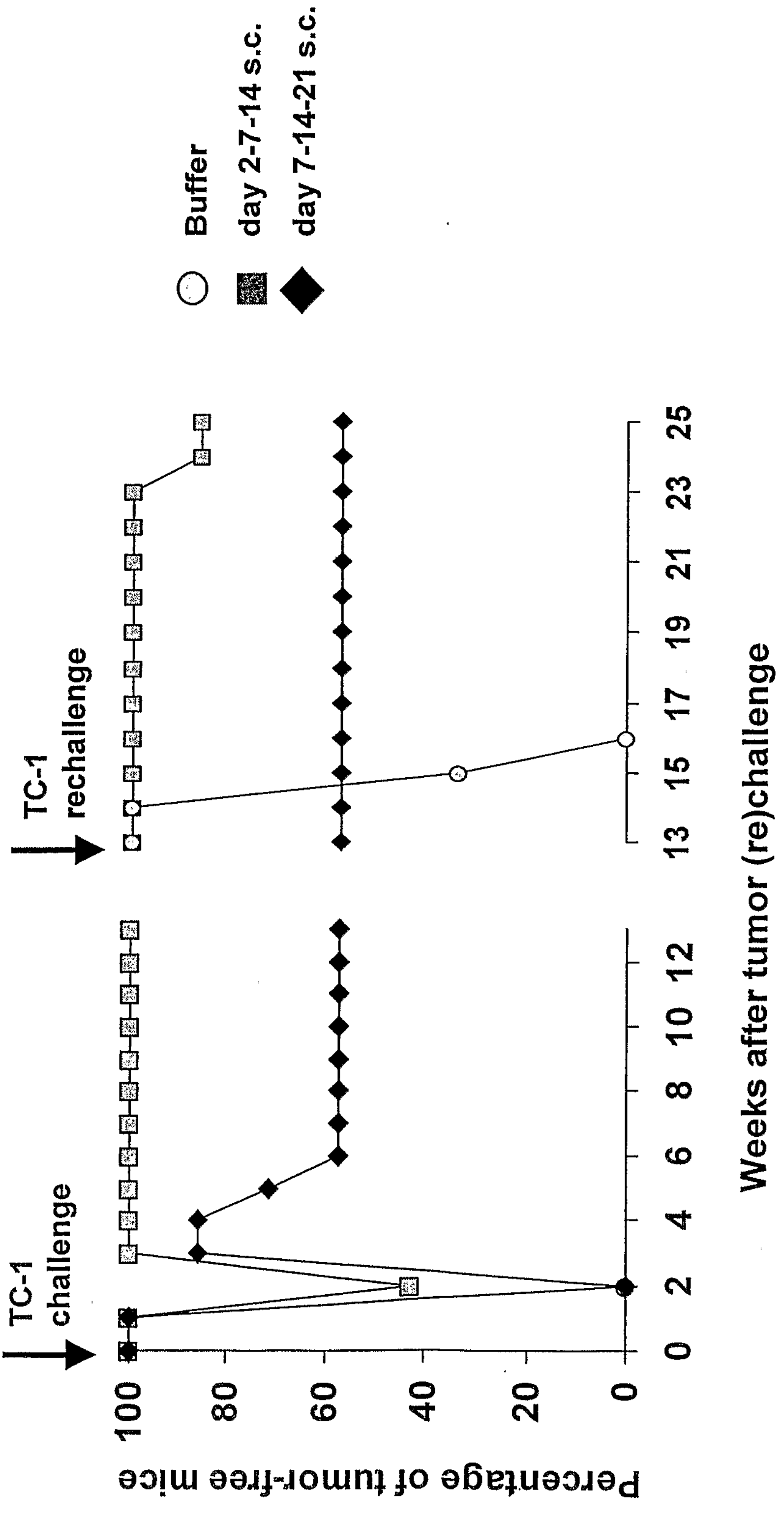

Buffer
day 2-7-14 s.c.
day 7-14-21 s.c.
TC-1 challenge
TC-1 rechallenge
Percentage of tumor-free mice
100
80
60
40
20
0
0
2
4
6
8
10
12
13
15
17
19
21
23
25
Weeks after tumor (re)challenge

FIGURE 21

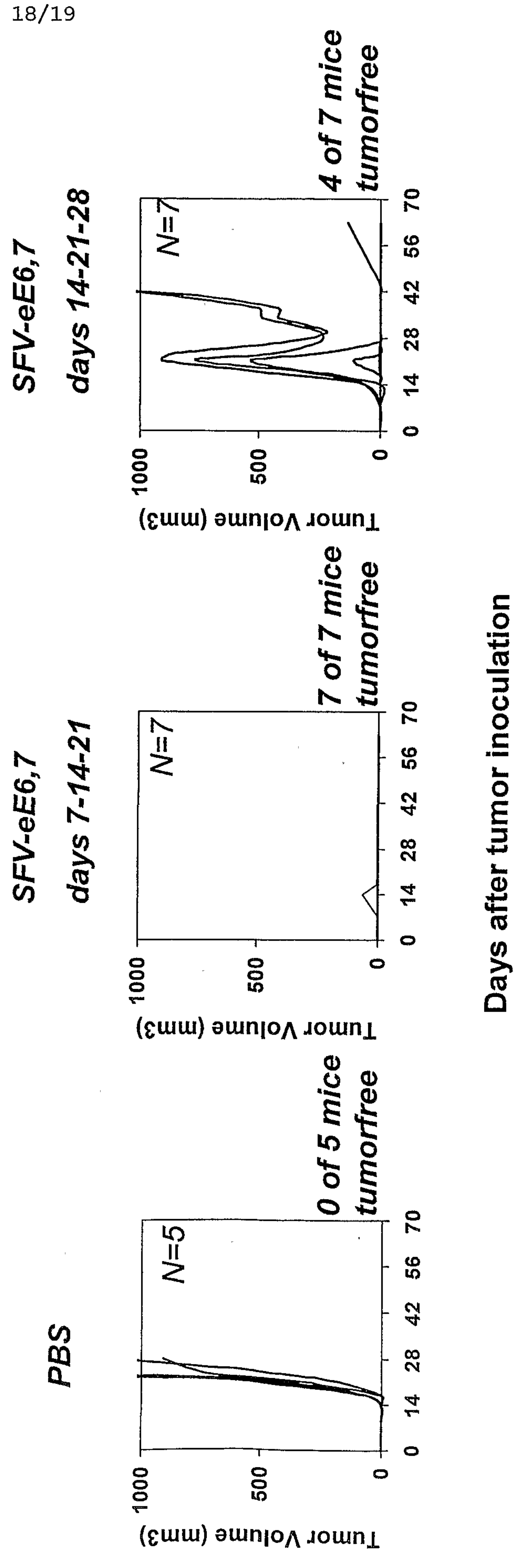
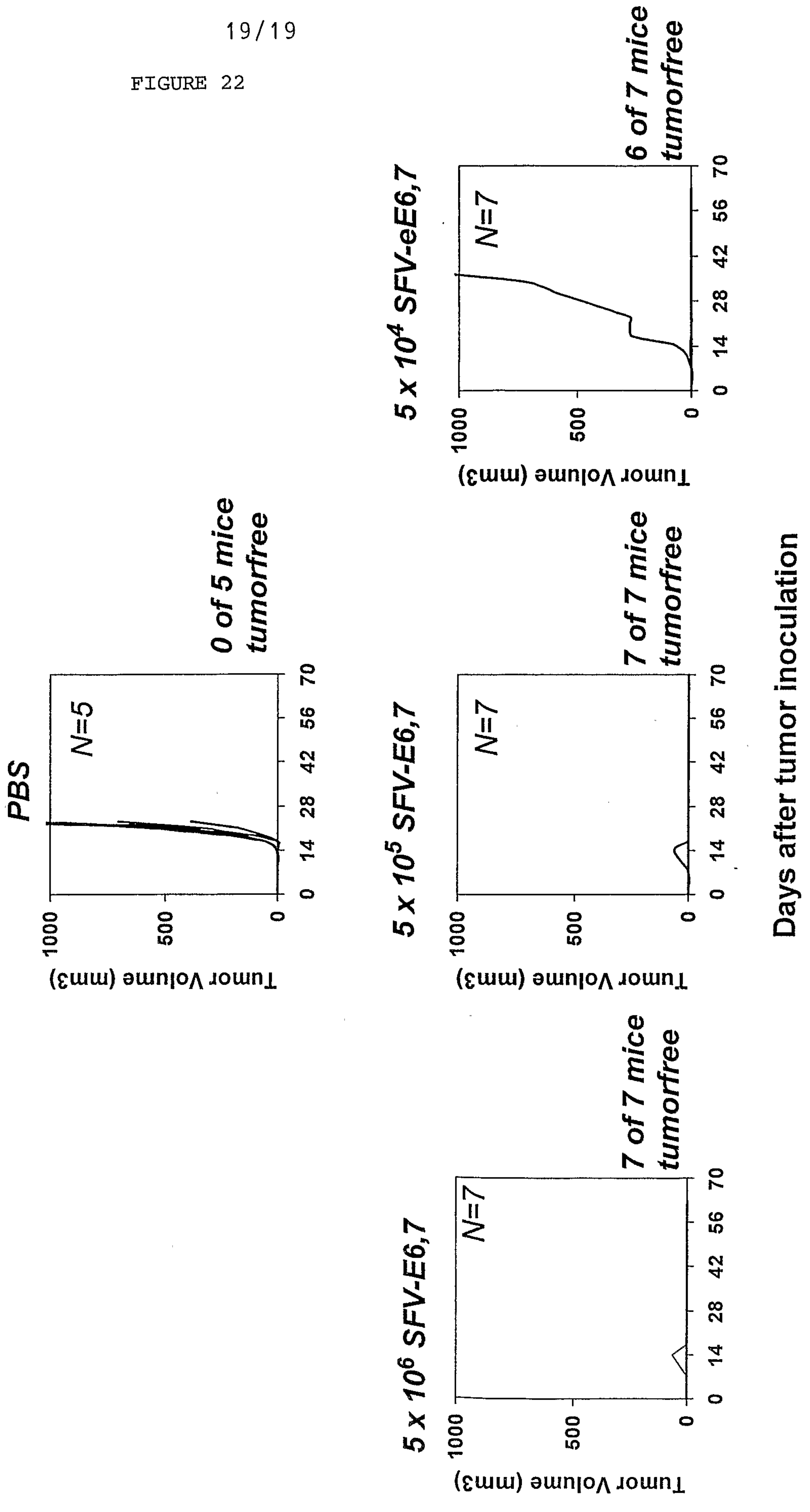

SFV-eE6,7
days 14-21-28
N=7
4 of 7 mice
tumorfree
SFV-eE6,7
days 7-14-21
N=7
7 of 7 mice
tumorfree
PBS
N=5
0 of 5 mice
tumorfree
Tumor Volume (mm3)
1000
500
0
0
14
28
42
56
70
Days after tumor inoculation

FIGURE 22


PBS
N=5
0 of 5 mice tumorfree
5 x 10^4 SFV-eE6,7
N=7
6 of 7 mice tumorfree
5 x 10^5 SFV-E6,7
N=7
7 of 7 mice tumorfree
5 x 10^6 SFV-E6,7
N=7
7 of 7 mice tumorfree
Tumor Volume (mm3)
1000
500
0
0
14
28
42
56
70
Days after tumor inoculation