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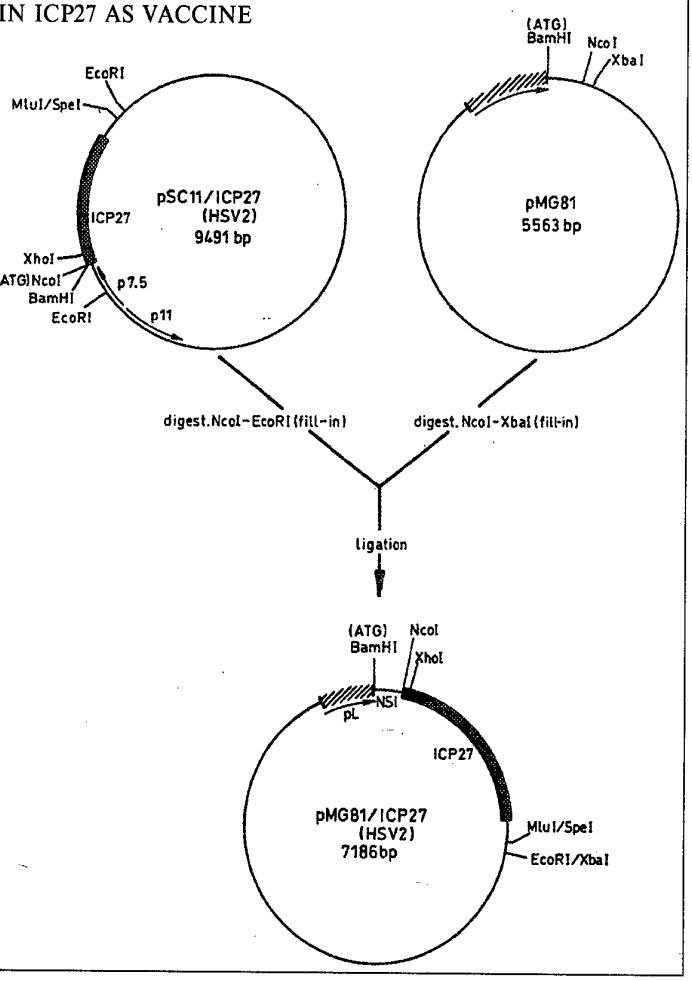
**(54) Title:** IMMEDIATE EARLY HSV-2 VIRAL PROTEIN ICP27 AS VACCINE

**(57) Abstract**

Immediate early HSV-2 viral protein ICP27 recognised by cytolytic T-lymphocyte (CTL) cells in humans, methods for preparation thereof and use in vaccine.



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# IMMEDIATE EARLY HSV-2 VIRAL PROTEIN ICP27 AS VACCINE

The present invention relates to therapeutic and prophylatic vaccines, novel antigens for use in such vaccine(s), methods for their preparation and their use in human medicine. In particular the present invention relates to antigens from Herpes Simplex (HSV) capable of stimulating a cytotoxic T lymphocyte response.

HSV causes lifelong infection and recurrent disease in man. There are two closely related serotypes of HSV, these are known as HSV-1 and HSV-2 respectively. In primary infections, after replication at a skin or mucosal site, the virus moves to the dorsal root ganglia and usually enters a latent phase. Reactivations then occur after appropriate stimuli, resulting in vesicles and ulcers at the mucocutaneous sites innervated by the ganglia. While neutralizing antibodies are shown to protect against primary infection and disease, their presence has no effect on the course or frequency of recurrent herpetic disease. T cell mediated immune responses, particularly of the delayed type hypersensitivity (DTH) or cytolytic (CTL) effector types have also been shown to protect against primary disease in mouse animal models. Furthermore, individuals with compromised T cell functions may undergo severe and sometimes life-threatening herpetic disease. These observations suggest a central role for effector T cell functions in control of herpes virus infections in man.

The major surface glycoproteins of Herpes Simplex Virus, gD and gC have been suggested for use in vaccines (EP 139 417 Genentech). These primarily stimulate a neutralising antibody response.

Since the mechanism of antigen recognition by CTL involves breakdown of native antigen into peptides, binding of the proteolytic fragments to MHC molecules and export of the complex to the cell surface, any virus coded polypeptide not just those that are integral membrane proteins like the glycoproteins, can be a potential target of T cell mediated responses. However since the HSV genome codes for several non structural proteins and internal virion proteins, in addition to external glycoproteins, this results in a large number of potential CTL targets and it is not known which protein would be the most relevant.

HSV infection is characterized by minimal presence of free virus. During latency and reactivation virus is mainly intracellular. Accordingly, recurrent disease is not prevented even by high levels of neutralizing antibodies and virus control depends on cell mediated immunity. In order to obtain protection by vaccination, it seems therefore desirable to induce not just an antibody response, but also CTL. An effective vaccine should prime CTL capable of acting as early as possible as soon as signs of reactivation of latent virus appear.

Previous studies have identified human CTL responses to various herpes

simplex structural components such as glycoproteins gD, gB (Zarling et al. 1986), but the relevance of these CTL for virus clearance is not known. Moreover, such CTL were HLA class II restricted, and although expression of class II molecules is induced in keratinocytes during HSV replication, it may occur too late to prevent the appearance of lesions.

In order to identify the most important CTL target antigens for prophylatic or therapeutic vaccine purposes, the present inventors have taken into consideration the HSV replicative cycle. After primary infection and during reactivation from a latent state in neuronal ganglia, HSV is mostly intracellular, with minimal exposure to neutralizing antibodies. However, the beginning of viral protein synthesis inside a cell that harbours viral genome will generate viral protein fragments that will be presented by MHC molecules on the surface of the cell, making it a target for CTL of the appropriate specificity. The replication cycle of HSV lasts about 18-20 hours and involves an ordered expression of $\alpha$ or immediate early (IE) $\beta$ or early (E) and $\gamma$ or late (L) gene products. Therefore early CTL attack and consequent lysis of the infected cells prior to late structural gene expression could prevent new virions being made and therefore prevent spread of the virus to neighbouring cells. In order to be most useful, CTL should detect the very first viral proteins that appear inside the cell after infection and reactivation.

We have analyzed the specificity of human HSV specific CTL towards immediate early viral protein ICP27. First, we investigated the CTL response in peripheral blood mononuclear cells (PBMC) from patients with herpetic genital lesions of varying clinical severity. We used autologous HSV-2 infected lymphoblasts as stimulators to induce HSV-2 specific HLA restricted CTL in mass and limiting dilution cultures. Strong responses were found in PBMC samples obtained days to weeks after the occurrence of lesions. The frequency of HSV-2 specific CTL ranged between 1/10000 and 1/36000.

Using vaccinia virus recombinant ICP27.VV the gene product was expressed in EBV transformed lymphoblastoid target cells for cytotoxicity assays. The recombinant infected target cells were recognized by a fraction of HSV-2 specific CTL induced by in vitro restimulation with HSV-2 infected lymphoblasts. This IE protein constitutes therefore a candidate component for HSV vaccines aimed at inducing CTL mediated immunity.

The present invention is therefore, directed towards an immediate early HSV-2-viral protein ICP27 that is recognised by cytolytic T lymphocyte (CTL) in humans. In particular an ICP 27 having substantially the sequence as shown in ID Sequence No.1 (protein sequence). The term substantially means at least 85% homologous, preferably 90 to 95% homologous, more preferably greater than 95% homologous.

Accordingly, the present invention provides a vaccine composition, for therapeutically or prophylactically treating HSV infections, comprising HSV-2, immediate early protein ICP27 or an immunologically active fragment thereof. The ICP27 protein may be expressed as a fusion protein or on a carrier such as a Hepatitis B surface antigen, or presented by a live bacterial carrier, such as listeria, shigella, BCG or Salmonella. Alternatively, the protein may be presented as in a live viral vector, such as vaccina, adenovirus or poliovirus. Alternatively the protein may be incorporated into an HSV light particle, as described in British patent application No. 91147140.0 and 9109763.4. ( published: WO 92/13943 and PCT GB92/00824).

Such forms of presentation of ICP27 form part of the invention. A preferred embodiment of the invention is a vaccinia recombinant which expresses an HSV-2 ICP27 protein or an immunologically active fragment thereof.

This is the first medical use ascribed to this protein, and accordingly in one aspect of the invention there is provided HSV-2 ICP27 for use in medicine.

ICP27 is an immediate early protein and its function in the virus is poorly understood, but it is known to be essential for viral replication and involved in virus genome transactivation [McCarthy, A.M., McMahan, L. Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. 63:18-27.; Rice, S.A., Su, L, Knipe, D.M. (1989). Herpes simplex virus alpha protein ICP27 possesses separable positive and negative regulatory activities. J. Virol. 83:3899-3407].

As used herein, an immunological fragment of ICP27 is a portion of the protein which is capable of eliciting a functional immunological response.

In animal models we have shown that inoculation with Recombinant vaccinia viruses expressing ICP27 after primary challenge with virulent virus, substantially reduces the frequency and severity of recurrent episodes of the disease.

A further aspect of the invention provides a process for the preparation of the ICP 27 HSV-2 protein or an immunogenic derivative thereof, which process comprises expressing DNA encoding said protein or derivative thereof in a recombinant host cell and recovering the product, and thereafter, optionally, preparing a derivative thereof.

A DNA molecule comprising such coding sequence eg as shown in ID Sequence No.2 or a fragment thereof forms a further aspect of the invention and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts et al in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by a combination of these techniques. With reference to ID sequence 2, the coding sequence for the mature protein ends at base no.1536.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50µl or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801.

Alternatively, the coding sequence can be derived from HSV-2 mRNA, using known techniques (e.g. reverse transcription of mRNA to generate a complementary cDNA strand), and commercially available cDNA kits.

The invention is not limited to the specifically disclosed sequence, but includes all molecules coding for the protein or an immunogenic derivative thereof, as described above.

DNA polymers which encodes mutants of the protein of the invention may be prepared by site-directed mutagenesis of the cDNA which codes for the protein by conventional methods such as those described by G. Winter et al in Nature 1982, **299**, 756-758 or by Zoller and Smith 1982; Nucl. Acids Res., **10**, 6487-6500, or deletion mutagenesis such as described by Chan and Smith in Nucl. Acids Res., 1984, **12**, 2407-2419 or by G. Winter *et al* in Biochem. Soc. Trans., 1984, **12**, 224-225.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et. al.*, Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide

sequence that encodes said HSV-2 ICP 27 protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the 16 kDa protein, or fragments thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of $CaCl_2$ (Cohen *et al*, Proc. Nat. Acad. Sci., 1973, **69**, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in

culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis **et al** and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as **E. coli** it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, absorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

Alternatively, the expression may be carried out in insect cells using a suitable vector such as the Baculovirus. In a particular aspect of this invention, the protein is expressed in Lepidoptera cells to produce immunogenic polypeptides. For expression of the protein in Lepidoptera cells, use of a baculovirus expression system is preferred. In such system, an expression cassette comprising the protein coding sequence, operatively linked to a baculovirus promoter, typically is placed into a shuttle vector. Such vector contains a sufficient amount of bacterial DNA to propagate the shuttle vector in *E. coli* or some other suitable prokaryotic host. Such shuttle vector also contains a sufficient amount of baculovirus DNA flanking the desired protein coding sequence so as to permit recombination between a wild-type baculovirus and the heterologous gene. The recombinant vector is then cotransfected into Lepidoptera cells with DNA from a wild-type baculovirus. The recombinant baculoviruses arising from homologous recombination are then selected and plaque purified by standard techniques. See Summers et al., **TAES Bull** (Texas Agricultural Experimental Station Bulletin) **NR 1555**, May, 1987.

A process for expressing the CS protein in insect cells is described in detail in USSN 287,934 of SmithKline RIT (WO/US 89/05550).

Production in insect cells can also be accomplished by infecting insect larvae. For example, the protein can be produced in *Heliothis virescens* caterpillars by feeding the recombinant baculovirus of the invention along with traces of wild type baculovirus and then extracting the protein from the hemolymph after about two days. See, for example, Miller *et al.*, PCT/WO88/02030.

The novel protein of the invention may also be expressed in yeast cells as described for the CS protein in EP-A-0 278 941.

Vaccina constructs can be made by methods well known in the art, see for example European Patent Application EP-083-286 Health Research Inc., Inventors Paoletti and Panicali. The construction of such a vaccinia construct is presented in more detail in the examples.

ICP27 has been shown by the present inventors, to be recognised by human HSV specific CTL induced by in vitro stimulation of PBMC (peripheral blood mononuclear cells) with HSV-2 infected cells. By using infected cells, as stimulator cells in vitro, viral epitopes which are synthesized in the cytoplasm, are preferentially presented by class I molecules. Thus the spectrum of effector cells stimulated in vitro by this approach will include both class I and class II restricted T cells.

This is in contrast with stimulation of primed PBMC using inactivated free virus, known to preferentially induce class II restricted effector CTLs, as the virus enters antigen presenting cells by endocytosis and is processed by the class II pathway. Neo-synthesis of antigen does not occur, and class I restricted presentation is less likely to occur.

The antigenic specificity of human CTL responses to HSV is highly relevant for an effective subunit vaccine, since HSV infection is characterised by the ability to establish latency and reactivate periodically. During latency and reactivation there is minimal exposure of free virus to antibodies as the virus is mainly maintained intracellularly.

In order to maximise the protective ability of a vaccine according to the invention, the vaccine may also preferably contain one or more other HSV proteins, other immediate early, early or late proteins capable of stimulating a CTL response in humans, such as gD or gC Vmw65, $RR_2$, ICPO or $ICP_4$. In particular, the vaccine may advantageously contain a truncated gD derivative from HSV-2 as described in EP 139 417 B. Also the vaccine may contain HSV-1 proteins or cocktails of variants of the same proteins where they exist.

Also the vaccine may contain HSV-1 proteins or cocktails of variants of the same proteins where they exist.

The vaccine of the present invention will preferably be adjuvanted. Known adjuvants will include alum (aluminium hydroxide) mycobacterium derived antigens such as Freunds complete or incomplete adjuvants, and muramyldipeptide (MDP) and derivatives, saponin type adjuvants such as QS21 (US Patent No 5057540) and the like. A particularly preferred adjuvant preparation is 3-0-de-acylated monophosphoryl lipid A (3D-MPL) which is commercially available from Ribi Immunochem and may be prepared according to the method of GB 2220211, or QS21 commercially available from Cambridge Biotech.

In such cases 3D-MPL and/or QS21 will be present in the range 10μg -

100µg, and preferably 25 - 50 µg per dose. The vaccine containing 3D-MPL or QS21 will typically be presented on alum or in an oil in water emulsion.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 2-100 µg, most preferably 4-40 µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

In addition to vaccination of persons susceptible to HSV infections, the pharmaceutical compositions of the present invention may be used to treat, immunotherapeutically, patients suffering from HSV infections, in order to prevent or significantly decrease recurrent herpes disease, frequency, severity and duration of episodes.

The rationale for immunotherapeutic use of the invention is that the frequency of HSV specific CTL, that exert an immune surveillance function against the virus, may physiologically decline with time after the last antigen-triggered expansion. Alternatively virus infection may not trigger a strong enough CTL response.

When low numbers of such CTL exist in the body, a reactivating HSV infection will have more chances to go through more rounds of viral replication before being detected by HSV specific T cells, resulting in larger clinically apparent herpetic lesions. However, if CTL levels are maintained at a given level by a suitable protocol of therapeutic vaccination, the time during which reactivating virus replicates unchecked will be kept to a minimum. This will have a beneficial effect in HSV infected individuals, eliminating or reducing the severity of clinically detectable recurrent lesions. This effect will be in addition to, and non exclusive of, the advantage provided by the specificity of CTL for an immediate early antigen, as referred to above.

A suitable protocol of therapeutic vaccination may be defined as a pharmacologically acceptable amount of vaccine preparation administered at regular

time intervals in HSV infected individuals, which results in elimination or reduced severity of previously occurring recurrent herpetic disease.

**EXAMPLE 1**

**HSV2 ICP27 EXPRESSION IN VACCINIA VIRUS**

1. INSERTION OF A SYNTHETIC POLYLINKER IN A VACCINIA EXPRESSION VECTOR

pRit13389 was obtained by insertion of a synthetic polylinker:

SC111 5' GGG AAA ACC ATG GAT CCA TGG CAG GTA CTA GTG TCG ACT AAC TAA CTA A 3'

SC112 3' CCC TTT TGG TAC CTA GGT ACC GTC CAT GAT CAC AGC TGA TTG ATT GAT T 5'

in the unique SmaI site of the PSC11 vaccinia virus expression vector ( Mackett M. et al., 1985. DNA cloning vol II, chap 7, a practical approach, Ed. DM Glover, ISBN 0-947946-19-5). See Figure 1.

2. **CONSTRUCTION FOR THE EXPRESSION OF HSV2 GENE ICP27**

A large part of the ICP27'S open reading frame (1481 bp) was cloned as a fragment of 1666 bps Xho1/M1u1 T4 polymerase treated. This fragment contains 185 bp downstream of the gene.

The missing 3' terminal region was reconstituted by a synthetic oligonucleotide:

UL541 5' CAT GGC TAC CGA CAT TGA TAT GCT AAT CGA CCT AGG ATT GGA CCT GTC CGA CAG CGA GC 3'

UL542 3' CG ATG GCT GTA ACT ATA CGA TTA GCT GGA TCC TAA CCT GGA CAG GCT GTC GCT CGA GCT 5'

These two fragments were ligated together in pRit13 389 between the Nco1 and Spe1 T4 polymerase treated sites. This construction is called pRit13 395, see figure 2.

3. **EXPRESSION IN VACCINIA VIRUS**

The expression cassette was used to transform vaccinia virus following the M. MACKETT et al. protocol except that 20 µg of plasmid DNA was used by experiment ( Mackett M. et al., 1985. ibid). CV1 cells (ATCC CCL70) and WR vaccinia virus strain were used for the transfection. Selection of recombinants was realized in rat 2 cells (ATCC CRL 1764) following Mackett *et al.* ß-galactosidase activity was detected as described in Chakrabarti S. *et al.*, Mol. Cell. Biol. 1985 5:3403-3409.

4. **RECOMBINANTS VACCINIA VIRUS**

Expression of recombinant genes products in vaccinia virus was tested by Western blot. The antibodies used for protein detections were obtained by immunizing rabbits with synthetic peptides (ICP27 aa2-12 ATDIDMLIDLG coupled to BSA carrier)].

Rabbit sera were diluted to 1/1000 to perform the Western blot.

**EXAMPLE 2**

**Expression of ICP27 (HSV2) in E. coli** Introduction: the Escherichia coli expression system used was plasmid pMG81, which is a derivative of pMG27N developed for the efficient production of proteins in E. coli (reference 1 below). Plasmid pMG27N is a derivative of the vector pAS1, which has been used to synthesize large quantities of numerous foreign proteins (ref. 2-9). The pMG27N plasmid, as pAS1, utilizes signals from l phage DNA to drive the transcription and translation of inserted foreign genes. The plasmid contains the l promoter PL; operator OL; two utilization sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided; the cII ribosome binding site including the cII translation initiation codon incorporated in an NdeI restriction site (ref. 1). Plasmid pMG1 has been constructed by inserting the 81 first amino acids of the NS1 coding region from influenza strain A/PR/8/34 cleaved from plasmid pAS1 EH/801 (ref. 4) by BamHI and NcoI into pMG27N digested by BamHI and SacI. A synthetic DNA linker resulting from the ligation of two synthetic oligonucleotides ($^{5'}$ATCCCGGGATAAAAACAACCAAGGTAATGGACA$^{3'}$ and $^{5'}$GCCCTATTTTTGTTGGTTCCATTACCTGTT$^{3'}$ was introduced between the NcoI and the SacI sites. pMG81 is a derivative of pMG1. pMG1 has been digested with BglII-PstI to remove the ampicillin resistance gene. The kanamycin resistance gene from transposon Tn903 (13-15) was isolated from plasmid pOTS207 (16) by a EcoRI-PstI digestion and ligated into pMG1 along with a synthetic DNA linker resulting from the ligation of two synthetic oligonucleotides ($^{5'}$AATTCGTACCTA$^{3'}$

and $^{5'}$GCATGGATCTAG$^{3'}$). The AR58 bacterial lysogen used for the production of the NS1-ICP27 protein is a derivative of the standard NIH E. coli K12 strain N99 (F- su-galK2 lacZ- thr-). It contains a defective phage lambda lysogen (galE::Tn10, l Kil- cI857 H1) which is Kil- (i.e. prevents the shut-off of host macromolecular synthesis), has a cI857 mutation (a temperature sensitive lesion in the cI repressor protein), and has the H1 deletion which removes the lambda phage right operon and the host bio, uvr3, and chlA loci (ref. 17). The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 (galE::Tn10, l Kil- cI857 H1) derivative. The introduction of the defective lambda lysogen into N99 was selected with tetracycline by virtue of the presence of a Tn10 transposon coding for tetracycline resistance in the adjacent galE gene. N99 and SA500 are E. coli K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

An important feature of this system is that plasmids containing the PL promoter are introduced into an E. coli lysogenic host to stabilize the plasmid DNA (ref. 3). Cloning into lysogens also precludes the synthesis of proteins that may be toxic to the cells (10). For these purposes, defective lambda phage lysogens are employed so that no phage production ever occurs. The integrated lambda phage DNA in the host genome directs the synthesis of a cI repressor protein which binds to the Ol operator on the plasmid and prevents the binding of RNA polymerase to the PL promoter. The inserted gene is therefore transcriptionally silent and no synthesis of the recombinant protein can occur. It is for this reason that during the cloning and growth of cells, there is no expression. However, if the defective lambda gene carries a temperature sensitive mutation in the cI gene (ref. 11), PL directed transcription can be regulated. Bacteria are grown in the absence of expression (30°C repressor is active) and then shifted to 42°C to inactivate the repressor and turn on the synthesis of the desired gene product. Construction of plasmid pMG81/ICP27Plasmid pMG81/ICP27, that expresses NS1-ICP27, has been constructed as follows (figure 3). A 1.7 kb fragment containing the ICP27 gene has been prepared by digesting plasmid pSC11/ICP27 with EcoR I, filling the protruding ends using the T4 DNA polymerase, and finally digesting the linear fragment with Nco I. Upon isolation on agarose gel and electroelution, this fragment has been ligated with plasmid pMG81 previously digested with Xba I, treated with the T4 DNA polymerase and then digested with Nco I. The ligation mixture has been transformed into Escherichia coli strain AR58. The transformants were selected onto solid medium containing kanamycin.

**Expression of NS1/ICP27** A strain AR58(pMG81/ICP27) has been incubated in 20 ml LB medium containing kanamycin, at 30°C up to an optical density (620 nm) of 0.6. The culture has then been incubated at 42°C for 3 hours.

Five ml of the culture have been taken at time 0, 30 min, 1 hour, 2 hours and 3 hours after induction. These samples have been submitted to an SDS-polyacrylamide gel electrophoresis. The proteins were then blotted onto a nitrocellulose membrane by electrotranfer. The ICP27-specific proteins were detected using rabbit α-ICP27 antibodies. Western blot analysis indicated the presence of a sharp band whose size was in agreement with the expected size of NS1-ICP27. Smaller ICP27-specific polypeptides were also detected with the rabbit antiserum: they probably represent degraded or incomplete product of the NS1-ICP27 gene.

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**EXAMPLE 3:** Recombinant Vaccinia Virus Immunizations

Induction of CTL specific for ICP27 by immunisation with recombinant vaccinia virus expressing ICP27 of HSV-1 in Balb/c mice has been described by Banks et al (1991) J. Virol. 65 : 3185 - 3191.

Similarly, vaccination with vaccinia recombinants expressing ICP27 of HSV-2 induces in Balb/c mice a specific CTL response and protects them against zosteriform spread of Wild type HSV n or HSV 2.

## Table 1

VACCINATION WITH VVICP27 (HSV1 OR HSV2) PROTECTS BALB/C MICE AGAINST ZOSTERIFORM SPREAD OF WILD TYPE HSV1 OR HSV2

| VACCINATION | CHALLENGE VIRUS | DOSE | % LESIONS * | % DEATHS * | CTL ** |
|---|---|---|---|---|---|
| VV ICP27 (HSV1) | HSV1 17+ | $10^5$ | 0 | 0 | |
| | | $10^4$ | 0 | 0 | |
| | | | | | + |
| | HSV2 MS | $10^5$ | 0 | 0 | |
| | | $10^4$ | 0 | 0 | |
| VV ICP27 (HSV2) | HSV1 17+ | $10^5$ | 0 | 0 | |
| | | $10^4$ | 0 | 0 | |
| | | $10^3$ | 0 | 0 | |
| | | | | | + |
| | HSV2 MS | $10^5$ | 0 | 0 | |
| | | $10^4$ | 0 | 0 | |
| | | $10^3$ | 0 | 0 | |
| CONTROL VV tk- | HSV1 17+ | $10^5$ | 100 | 100 | |
| | | $10^4$ | 100 | 100 | |
| | | $10^3$ | 100 | 100 | |
| | | | | | - |
| | HSV2 MS | $10^5$ | 100 | 100 | |
| | | $10^4$ | 100 | 100 | |
| | | $10^3$ | 100 | 100 | |

* Groups of Balb/c mice (15 mice/group) were immunized with $10^7$ pfu of ICP27.VV in the footpad and challenged 2 weeks later with wild type HSV-1 ($17^+$) or HSV-2 (MS). The occurrence of zosteriform lesions and death was then recorded.

** Balb/c mice were immunized with $10^7$ pfu of ICP27.VV in the footpad. Draining popliteal lymph nodes were removed 5 days later for CTL assays, without in vitro restimulation, in order to evaluate the primary response.

**EXAMPLE 3:** Recognition of ICP 27 by human CTL

**Materials and methods:** **Patients**

Blood samples from patients attending the sexually transmitted disease clinic of the Topical Medicine Institute, Antwerp, were collected by venipuncture into

heparinised tubes. Patients had genital herpetic lesions of varying clinical severity and differed in recurrent disease patterns. One asymptomatic sexual partner of a patient with recurrent disease was included in the study.

**Viruses** **Herpes simplex virus.** The HG52 strain of herpes simplex virus type 2(HSV-2) used in these experiments was kindly provided by Prof. Subak-Sharpe (MRC, Glasgow, U.K.). The virus was grown in BHK21 cells infected at a multiplicity of infection (m.o.i.) of 0.003 plaque forming units (p.f.u.) per cell. The cells were harvested at 5-7 days after infection, disrupted by freezing/thawing and sonicated. The virus titre was determined by plaque assay on BHK21 cells.

**ICP27 vaccinia recombinant** was produced as herein described.

**Medium** PBMC cultures were grown in RPMI 1640 (Gibco, Ghent, Belgium) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS)(Flow laboratories, Irwine, Scotland), 2 x $10^{-3}$ M L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 x $10^{-5}$ M mercaptoethanol, 1% MEM non-essential amino acids (Gibco), 1 x $10^{-3}$ M sodium pyruvate MEM (Gibco).

**Cells.** Peripheral blood mononuclear cells (PBMC) were obtained from blood by separation on a Lymphoprep (Nycomed, Oslo, Norway) density gradient (Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, Suppl. 97, 77). PBMC were frozen in 10% DMSO 90% FCS (v/v) and thawed just before use as responder cells.

An aliquot of PBMC from each patient was used to derive lymphoblastoid cell lines (LCL) by transformation with Epstein-Barr virus (EBV obtained from culture supernatants of the persistently infected marmoset cell line B-95.8, as described (Walls, E.V. and Crawford, D.H. (1987)) Generation of human B lymphoblastoid cell lines using Epstein - Barr virus. In : Lymphocytes, a practical approach, Klaus, G.G.B. (editor) pp.149 - 162. The LCL were used as target cells in cytotoxicity assays.

PHA-activated lymphoblasts for use as stimulator cells were prepared by culturing 5 x $10^6$ PBMC with 4 µg/mL PHA-P (Sigma) for 72 hours at 37°C and with 5 U.mL rIL2 (Boehringer) for 7 more days at 37°C. The lymphoblasts were then infected with HSV-2 (m.o.i.= 10) for 16 hours at 37°C and treated with 1% formaldehyde in PBS for 20 min at 4°C.

**Mass cultures** PBMC were thawed and stimulated in 24-well plate cultures containing 2 x $10^6$ responder cells and 5 x $10^5$ stimulator cells, in the presence of 1 U/mL human recombinant IL-2 (rIL-2, Boehringer Mannheim) and 5% (v/v) supernatant from PHA activated lymphoblasts. Cultures were fed every 3-4 days with medium supplemented with 5 U/mL rIL-2 and 5% PHA-blast supernatant. Cultures were re-stimulated on day 10 and tested on day 20 for cytolytic activity.

**Limiting dilution cultures.** PBMC were thawed and distributed into 96-well round-bottom plates. The number of responder cells per well ranged between $10^3$ and 4 x $10^4$ and 24 to 32 wells were set up for each input cell concentration. Autologous stimulator cells (5 x $10^4$.well) were added to all wells. Control wells without responder cells were included. Cultures received 1 U/ml rIL-2 and 5% (v/v) PHA-blast supernatant at the onset, and were fed with 5U/ml rIL-2 and 5% (v/v) PHA-blast supernatant every 4-6 days. Equal aliquots from each individual culture were tested on day 14-21 in a chromium release assay against 3 different target cell types; autologous LCL infected with HSV-2, psC11.VV and ICP27.VV.

**Cytotoxicity assays** LCL target cells were infected with HSV-2, psC11.VV or ICP27.VV (m.o.i.=10) for 1 hour at 37°C, washed and labelled with 500 µCi of $^{51}Cr$ (Medgenix, Fleurus, Belgium) for 1 hour at 37°C. Target cells were then washed twice, incubated on ice for 30 min, washed once and 2 x $10^3$ cells per well were distributed into the wells containing responder cells and control wells containing medium or Triton X-100 3% in water (spontaneous release and maximum release, respectively). Effector and target cell mixtures were incubated for 4 hours at 37°C in a total of 200 µL, then 100 µL of supernatant were harvested and released $^{51}Cr$ counted. Results were expressed as % specific lysis according to the formula:

$$\% \text{ specific lysis} = \frac{\text{(experimental - spontaneous release)}}{\text{(total - spontaneous release)}} \times 100$$

**CTL frequency determinations and scatter plots.** Responder frequencies were calculated using the maximum likelihood method described by Fazekas de St. Groth (Fazekas de St. Groth, S. (1982). The evaluation of limiting dilution assays. J. Imm. Meth. 49:R11-R23). For each target cell type, wells were scored as positive if the % specific lysis was higher than the cut-off value defined as the average of control wells without responder cells + 3 standard deviations. The frequency estimates of HSV-2 and ICP27 specific CTL were obtained after exclusion of any wells that scored positive on control targets (psC11.VV infected).

Scatter plots were used to evaluate the specificity of cytolytic activity in limiting dilution cultures. Individual wells were split into equal volumes and tested for lysis of target cells infected with HSV-2, psC11.VV, or ICP27.VV. Each well thus produced multiple values of % specific lysis. Paired values for selected target cell types were used as coordinates to represent each well in a 2-dimensional cartesian plot. By plotting the cut-off values for each target cell type, the plot was partitioned into 4 sectors: wells negative on both targets, positive on both, and exclusively positive on one but not the other target.

**Results** **Mass culture induction of HSV-2 specific CTL.** PBMC from 10 patients with various clinical symptoms of genital herpes were stimulated in mass cultures as described in materials and methods. The cultures were then tested for HSV-2 specific cytolytic activity in $^{51}Cr$ release assays, using autologous and heterologous LCL target cells as HLA restriction controls.

Representative results are shown in Table 2. Six patients like IO2 had HSV-2 specific HLA-restricted effectors, while in four patients, like 109, no such activity could be demonstrated. All patients where CTL could not be induced had active lesions at the time when the blood sample was taken. Only one patient with ongoing lesions had detectable CTL specific for HSV-2 (table 2).

**Frequency of HSV-2 specific CTL.** In order to estimate the frequency of HSV-2 specific CTL, varying numbers of patient PBMC were cultured in the presence of a constant number of autologous stimulator cells (HSV-2 infected PHA-blasts prepared as described in materials and methods). Each well was tested for lysis of autologous LCL infected with HSV-2 or psC11.VV as negative control. Out of 14 patients tested in this way, 3 (108, 114 and K01) had high frequencies of effectors that lysed psC11.VV infected target cells, and were therefore not considered. In 7 patients (107, 109, 111, 114, 116, 118, 120) the frequency of HSV-2 specific CTL ranged between 1/10000 and 1/36000 (see for an example patient 111, table 3), while the remaining 3 other patients (101, 110, 112,) had frequencies lower than 1/89000 (table 3). The correlation that was seen with mass cultures between detectable CTL activity and time of appearance of lesions is no longer evident: patients that were negative for CTL in mass cultures show CTL detectable in limiting dilution cultures. No obvious correlation seems to exist between number of recurrences per year and frequency of CTL. Detection of CTL activity in limiting dilution cultures appeared more sensitive than in mass cultures. For instance, patient 109 appeared negative in mass cultures, but HSV-2 specific CTL were found in limiting dilution cultures (compare table 2 and table 3).

**Viral antigens recognized by HSV-2 specific CTL.** In order to evaluate the role of ICP27 in CTL recognition, limiting dilution cultures of patient PBMC stimulated with HSV-2 infected stimulators were split 4-ways and 3 aliquots from each well tested on autologous LCL infected with HSV-2, psC11 vaccina virus.VV or ICP27.VV. (Table 4) Out of 7 patients with high frequencies of HSV-2 specific CTL, two (116 and 118) had ICP27 specific CTL (frequencies of 1/22000 and 1/50000). In patient 118, ICP27 seems to be the main HSV-2 antigen recognized by CTL. Intermediate frequencies of CTL specific for ICP27 antigen occurred in patients like 107. Patient 109 had very low frequencies of CTL specific for ICP27.

**Table 2.**

Correlation between detection of HSV-2 specific CTL in mass cultures and time since appearance of lesions.

| Patient | Patient history* | | HSV-2 specific CTL |
|---|---|---|---|
| | Time since last episode | Recurrences per year | |
| 102 | 8 days | primary infection | yes |
| 107 | ongoing | 8 | no*** |
| 108 | ongoing | 7 | yes |
| 109 | ongoing | 3 | no*** |
| 112 | 1 month | 6 | yes |
| 114 | 1 year | 1 | yes |
| 116 | asymptomatic** | none | yes |
| 117 | ongoing | 12 | no*** |
| 118 | ongoing | 6 | no*** |
| K01 | 10 days | >10 | yes |

Patient PBMC were stimulated in mass cultures with HSV-2 infected stimulator cells and tested for cytolytic activity on uninfected or HSV-2 infected autologous or heterologous LCL targets.

* Disease history was reported by patients. The number of recurrences per year is normalized to one year on the basis of the number of recurrences in variable length intervals that the patient reported.

** This patient is the asymptomatic sexual partner of a patient with recurrent lesions.

*** These patients had detectable CTL in limiting dilution cultures.

**Table 3.**

Frequency of HSV-2 specific CTL

| Patient | Frequency (1/n) of CTL recognizing target cells infected by: | | Patient history* | |
|---|---|---|---|---|
| | psC11.VV | HSV-2 | Time since last episode | Recurrences per year |
| 101 | 36000 | 107000 | 6 months | 12 |
| 107 | 73000 | 23000 | ongoing | 8 |
| 108 | 13000 | 10000 | ongoing | 7 |
| 109 | 141000 | 36000 | ongoing | 3 |
| 110 | 43000 | too low | ongoing | persistent lesions |
| 111 | 68000 | 22000 | 2 weeks | 10 |
| 112 | 41000 | 89000 | 1 month | 6 |
| 114 | 14000 | 45000 | 1 year | 1 |
| 116 | too low | 24000 | - | asymptomatic |
| 118 | 48000 | 18000 | ongoing | 6 |
| 120 | 67000 | 28000 | 1 month | primary infection |
| K01 | 20000 | 7000 | 10 days | >10 |

PBMC were stimulated in limiting dilution cultures with autologous HSV-2 infected lymphoblasts.

Individual wells were split and tested on target cells infected with HSV-2 or psC11.VV.

* Disease history was reported by patients. The number of recurrences per year is normalized to one year on the basis of the number of recurrences in variable length intervals that the patient reported.

**Table 4.**

Frequency of ICP27 specific CTL compared to that of CTL recognizing HSV-2.

| Patient | Frequency (1/n) of CTL recognizing target cells infected by: | | Patient history | |
|---|---|---|---|---|
| | HSV-2 | ICP27.VV | Time since last episode | Recurrences per year |
| 107 | 23000 | 70000 | ongoing | 8 |
| 109 | 36000 | 194000 | ongoing | 3 |
| 111 | 22000 | 179000 | 2 weeks | 10 |
| 116 | 24000 | 5000 | no lesions | none |
| 117 | 10000 | 108000 | ongoing | 12 |
| 118 | 18000 | 22000 | ongoing | 6 |
| 120 | 28000 | 198000 | 1 month | primary lesion |

PBMC from patients with good responses to HSV-2 (CTL frequencies between 1/10000 and 1/36000) were evaluated for recognition of ICP27. The frequencies were calculated after exclusion of wells with lytic activity on control target cells infected with psC11.VV.

* Disease history was reported by patients. The number of recurrences per year is normalized to one year on the basis of the number of recurrences in variable length intervals that the patient reported.

We show herein for the first time that ICP27 HSV-2 is recognized by human HSV specific CTL induced by in vitro stimulation of PBMC with HSV-2 infected cells. ICP27 is a 63 Kdalton polypeptide coded by one of the five alpha genes that are expressed first upon infection, and reaches peak synthesis at 2-4 hours. ICP27 has regulatory functions and is probably essential for expression of late genes. In a subset of 7 patients with frequencies of HSV-2 specific CTL ranging between 1/10000 and 1/36000, two patients had frequencies of ICP27 specific CTL of 1/22000 and 1/5000. These frequencies are calculated after exclusion of all cultures scoring positive on control target cells, and constitute therefore minimal estimates.

These results show that an important share of the human response to herpes simplex virus is directed against a non-virion polypeptide. For each of the patients that did recognize this antigen, the response constituted a major component of the total response to HSV-2, although we cannot exclude that underrepresentation of late antigens may have occurred in the conditions used to infect target cells with HSV-2.

CLAIMS

1. Immediate early HSV-2 viral protein ICP27 that is recognised by cytolytic T lymphocyte (CTL) in humans having substantially the sequence shown in Seq. ID 1 or an immunologically or antigenically equivalent derivative or fragment thereof

2. A protein according to claim 1 wherein the protein is expressed as a fusion protein or on a carrier.

3. A protein according to claim 1 or 2 wherein the protein is presented by a live bacterial vector or a viral vector or is incorporated into an HSV light particle.

4. A protein according to claim 3 wherein the viral vector is vaccinia.

5. A DNA sequence having the sequence depicted in sequence ID 2 or a fragment thereof, or a DNA sequence which hybridises to said sequence and which codes for a protein having the biological activity of HSV-2 ICP27.

6. An expression vector comprising DNA as defined in claim 5.

7. HSV-2 ICP27 for use in medicine.

8. A process for the preparation of a protein according to any one of claims 1 to 4 which comprises:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said HSV-2 ICP 27 protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

9. A vaccine composition for therapeutically or prophylactically treating HSV infections, comprising a protein as defined in any one of claims 1 to 4 in admixture with a suitable carrier.

10. A vaccine composition according to claim 9 which comprises 3D-MPL or QS21.

11. A method of treating a human or animal susceptible to HSV infections comprising administering an effective amount of a vaccine as defined in claims 9 or 10.

**Sequence No. 1**

```
  1  MATDIDMLID LGLDLSDSEL EEDALERDEE GRRDDPESDS SGECSSSDED
 51  MEDPCGDGGA EAIDAAIPKG PPARPEDAGT PEASTPRPAA RRGADDPPPA
101  TTGVWSRLGT RRSASPREPH GGKVARIQPP STKAPHPRGG RRGRRRGRGR
151  YGPGGADSTP KPRRRVSRNA HNQGGRHPAS ARTDGPGATH GEARRGGEQL
201  DVSGGPRPRG TRQAPPPLMA LSLTPPHADG RAPVPERKAP SADTIDPAVR
251  AVLRSISERA AVERISESFG RSALVMQDPF GGMPFPAANS PWAPVLATQA
301  GGFDAETRRV SWETLVAHGP SLYRTFAANP RAASTAKAMR DCVLRQENLI
351  EALASADETL AWCKMCIHHN LPLRPQDPII GTAAAVLENL ATRLRPFLQC
401  YLKARGLCGL DDLCSRRRLS DIKDIASFVL VILARLANRV ERGVSEIDYT
451  TVGVGAGETM HFYIPGACMA GLIEILDTHR QECSSRVCEL TASHTIAPLY
501  VHGKYFYCNS LF
```

**Sequence No. 2**

```
   1  ATGGCTACCG ACATTGATAT GCTAATCGAC CTAGGATTGG ACCTGTCCGA
  51  CAGCGAGCTC GAGGAGGACG CTCTGGAGCG GGACGAGGAG GGCCGCCGCG
 101  ACGACCCCGA GTCCGACAGC AGCGGGGAGT GTTCCTCGTC GGACGAGGAC
 151  ATGGAAGACC CCTGCGGAGA CGGAGGGGCG GAGGCCATCG ACGCGGCGAT
 201  TCCCAAAGGT CCCCCGGCCC GCCCCGAGGA CGCCGGCACC CCCGAAGCCT
 251  CGACGCCTCG CCCGGCAGCG CGGCGGGGAG CCGACGATCC GCCACCCGCG
 301  ACCACCGGCG TGTGGTCGCG CCTCGGGACC AGGCGGTCGG CTTCCCCCCG
 351  GGAACCGCAC GGGGGGAAGG TGGCCCGCAT CCAACCCCCG TCGACCAAGG
 401  CACCGCATCC CCGAGGCGGG CGGCGAGGTC GCCGCCGGGG CCGGGGTCGA
 451  TACGGCCCCG GCGGCGCCGA CTCCACACCA AAACCCCGCC GGCGCGTCTC
 501  CAGAAACGCC CACAACCAAG GGGGTCGCCA CCCCGCGTCG GCGCGGACGG
 551  ACGGCCCCGG CGCCACCCAC GGCGAGGCGC GGCGCGGAGG GGAGCAGCTC
 601  GACGTCTCCG GGGGCCCGCG GCCACGAGGC ACGCGCCAGG CCCCCCCTCC
 651  GCTGATGGCG CTGTCCCTGA CCCCCCCGCA CGCGGACGGC CGCGCCCCGG
 701  TCCCGGAGCG AAAGGCGCCC TCTGCCGACA CCATCGACCC CGCCGTTCGG
 751  GCGGTTCTGC GATCCATATC CGAGCGCGCG GCGGTCGAGC GCATCAGCGA
 801  AAGCTTTGGA CGCAGTGCCC TGGTCATGCA AGACCCCTTT GGCGGGATGC
 851  CGTTTCCCGC CGCGAACAGC CCCTGGGCTC CCGTGCTGGC CACCCAAGCG
 901  GGGGGGTTTG ACGCCGAGAC CCGTCGGGTT TCCTGGGAAA CCCTGGTCGC
 951  TCACGGCCCG AGCCTCTACC GCACATTCGC AGCCAACCCG CGGGCCGCGT
1001  CGACAGCCAA GGCCATGCGC GACTGCGTGC TGCGCCAGGA AAATCTCATC
1051  GAGGCCCTGG CGTCCGCGGA TGAGACGCTG GCGTGGTGCA AGATGTGCAT
1101  TCACCACAAT CTGCCGCTCC GCCCCCAGGA CCCTATCATC GGAACGGCGG
1151  CCGCCGTGCT GGAAAACCTC GCCACGCGCC TGCGCCCCTT TCTGCAGTGC
1201  TACCTGAAGG CCCGAGGCCT GTGCGGGCTG GACGACCTGT GCTCGCGGCG
1251  ACGCCTGTCG GACATTAAGG ATATTGCCTC CTTTGTGTTG GTCATCCTGG
1301  CCCGCCTCGC CAACCGCGTC GAGCGCGGCG TGTCGGAGAT CGACTACACG
1351  ACCGTGGGGG TTGGGGCCGG CGAGACGATG CACTTTTACA TCCCGGGGGC
```

**Sequence No. 2 contd.**

1401 CTGCATGGCG GGTCTCATTG AAATACTGGA CACGCACCGC CAGGAGTGTT

1451 CCAGTCGCGT GTGCGAGCTG ACGGCCAGTC ACACTATCGC CCCCTTATAT

1501 GTGCACGGCA AATACTTCTA CTGCAACTCC CTATTTTAGG CAAGAATAAA

1551 CATATTGACG TCAACCCAAG TGGTTCCGTG TGATGTTCTT GGCGCGCGCG

1601 GCGGGTGGGG CGGAGACTCC GGGGCGATGC CGGCGTGCGC GTGGGAGGAG

1651 GGCGATGACC CACCGGATAA ATGTGGGGCC CCGGCCCGGC CCGCTTCATA

1701 GCGCGTCCAG GAACTCACGG CAGACGCGT

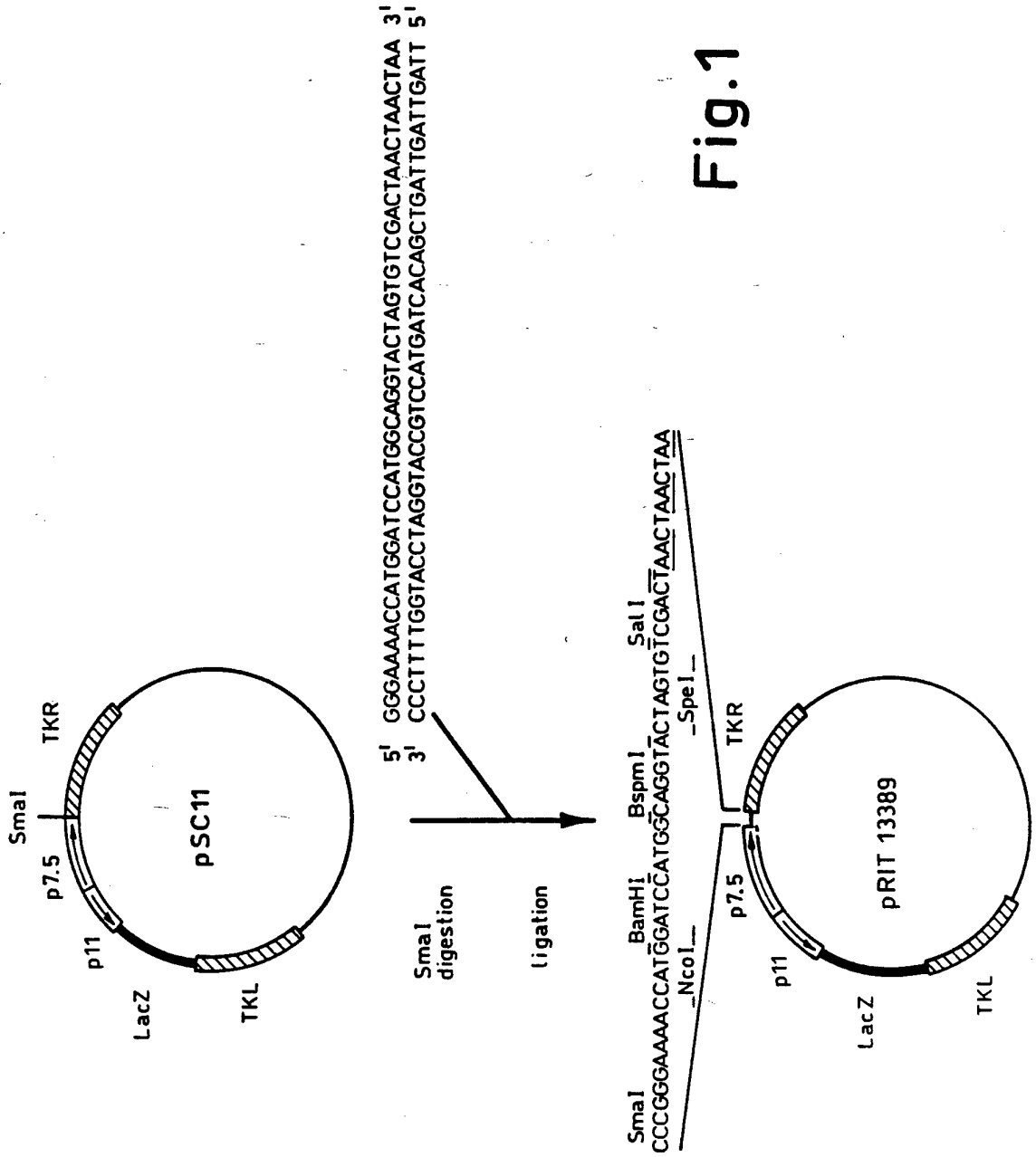
SmaI
TKR
p7.5
p11
LacZ
TKL
pSC11
SmaI digestion
5' GGGAAAACCATGGATCCATGGCAGGTACTAGTGTCGACTAACTAACTAA 3'
3' CCCTTTTGGTACCTAGGTACCGTCCATGATCACAGCTGATTGATTGATT 5'
ligation
SmaI
BamHI
BspmI
SalI
NcoI
SpeI
TKR
p7.5
p11
LacZ
TKL
pRIT 13389

Fig.1

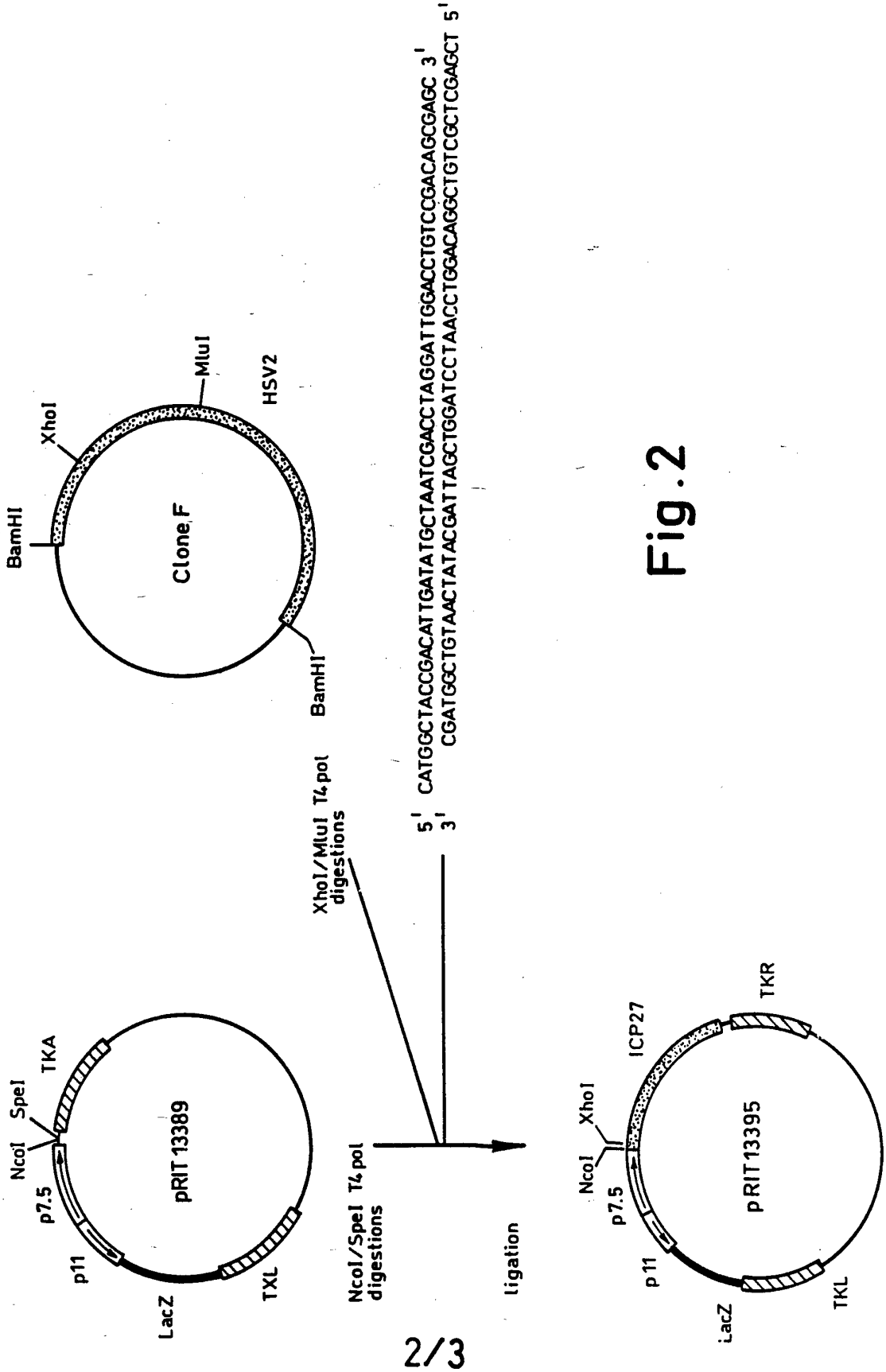
BamHI
XhoI
MluI
HSV2
Clone F
BamHI
5' CATGGCTACCGACATTGATATGCTAATCGACCTAGGATTGGACCTGTCCGACAGCGAGC 3'
3' CGATGGCTGTAACTATACGATTAGCTGGATCCTAACCTGGACAGGCTGTCGCTCGAGCT 5'
XhoI/MluI T4 pol digestions
NcoI/SpeI T4 pol digestions
ligation
NcoI
SpeI
TKA
p7.5
pRIT 13389
p11
LacZ
TXL
NcoI
XhoI
ICP27
TKR
p7.5
pRIT 13395
p11
LacZ
TKL

Fig.2

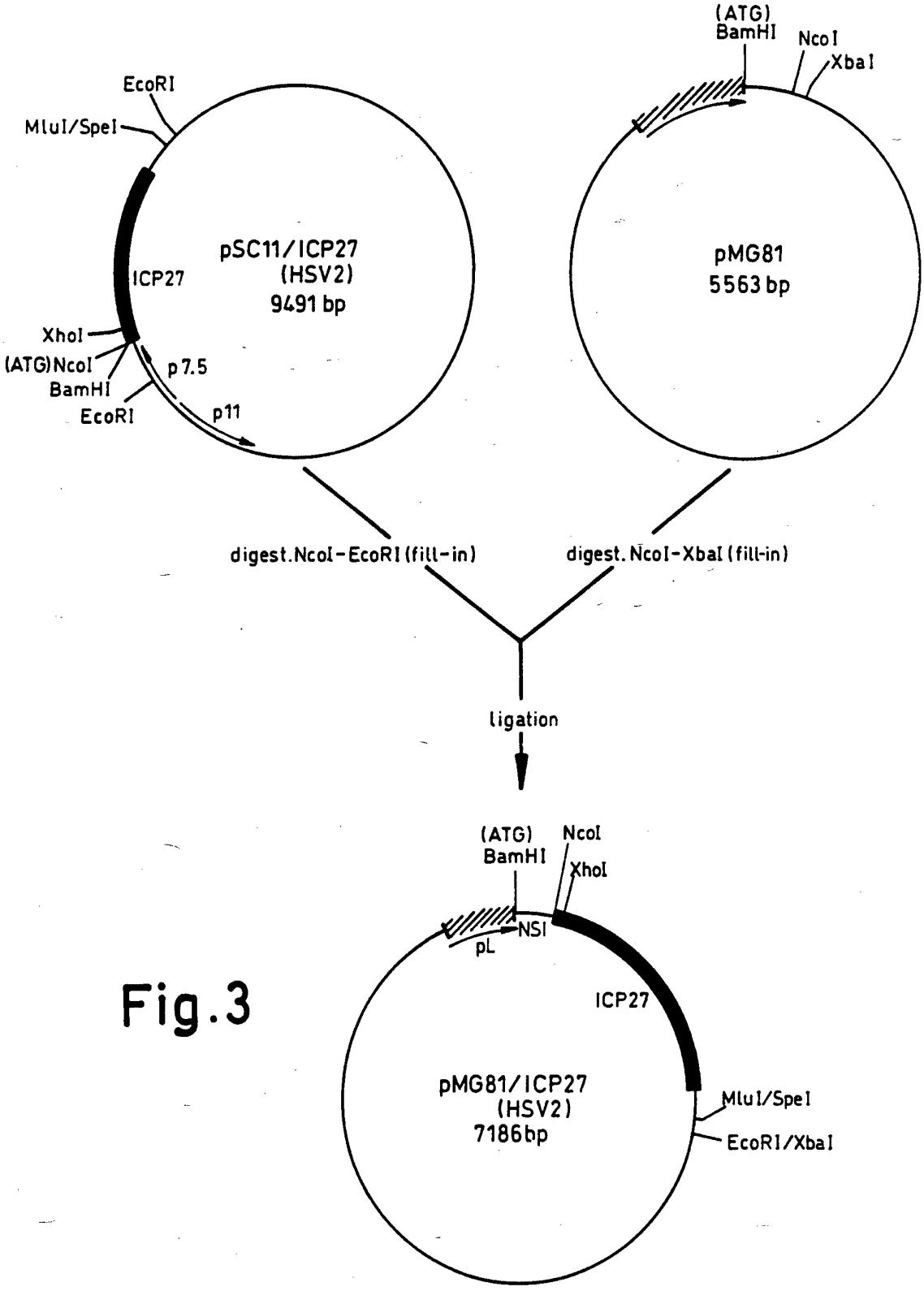
(ATG)
BamHI
NcoI
XbaI
EcoRI
MluI/SpeI
pSC11/ICP27
(HSV2)
9491 bp
ICP27
pMG81
5563 bp
XhoI
(ATG)NcoI
BamHI
EcoRI
p7.5
p11
digest.NcoI-EcoRI (fill-in)
digest. NcoI-XbaI (fill-in)
ligation
(ATG)
BamHI
NcoI
XhoI
NSI
pL
ICP27
pMG81/ICP27
(HSV2)
7186bp
MluI/SpeI
EcoRI/XbaI

Fig.3

# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/01525

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/38; C07K13/00; C12N15/62; C12N15/86
C12N7/04; A61K39/245; C12N15/44

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C07K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| X | JOURNAL OF GENERAL VIROLOGY<br>vol. 72, no. 12, December 1991,<br>pages 3057 - 3075<br>MCGEOCH, D.J. ET AL. 'Comparative sequence analysis of the long terminal repeat regions in the genomes of herpes simplex viruses types 1 and 2'<br>see page 3059, column 1, line 5 - page 3065, column 1, line 10; figures 1,3; table 2<br>--- | 1,5 |

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 08 OCTOBER 1993 | 14. 10. 93 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | CHAMBONNET F.J. |

# INTERNATIONAL SEARCH REPORT

nternational application No.
PCT/EP 93/01525

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claim 11 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.