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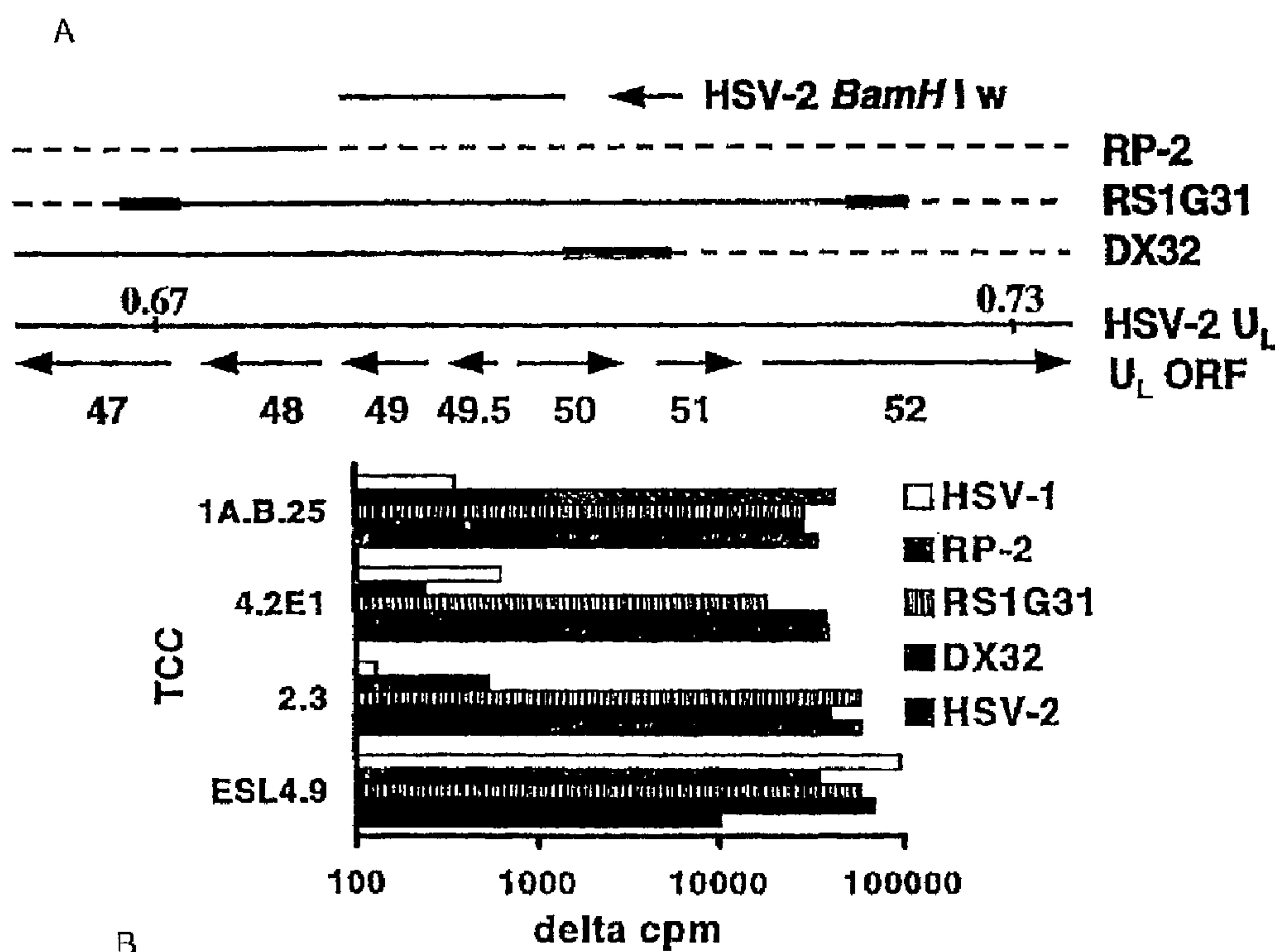
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(54) Titre : ANTIGENES DU VIRUS DE L'HERPES SIMPLEX (HSV) IMPORTANTS D'UN POINT DE VUE
IMMUNOLOGIQUES ET PROCEDES D'UTILISATION CORRESPONDANTS

(54) Title: IMMUNOLOGICALLY SIGNIFICANT HERPES SIMPLEX VIRUS ANTIGENS AND METHODS FOR USING
SAME



(57) Abrégé/Abstract:

The invention provides HSV antigen that are useful for the prevention and treatment of HSV infection. Disclosed herein are epitopes confirmed to be recognized by T-cells derived from herpetic lesions. T-cells having specificity for antigens of the invention have demonstrated cytotoxic activity against cells loaded with virally-encoded peptide epitopes, and in many cases, against cells infected with HSV. The identification of immunogenic antigens responsible for T-cell specificity provides improved anti-viral therapeutic and prophylactic strategies. Compositions containing antigens or polynucleotides encoding antigens of the invention provide effectively targeted vaccines for prevention and treatments of HSV infection.

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Abstract

The invention provides HSV antigen that are useful for the prevention and treatment of HSV infection. Disclosed herein are epitopes confirmed to be recognized by T-cells derived from herpetic lesions. T-cells having specificity for antigens of the invention have demonstrated cytotoxic activity against cells loaded with virally-encoded peptide epitopes, and in many cases, against cells infected with HSV. The identification of immunogenic antigens responsible for T-cell specificity provides improved anti-viral therapeutic and prophylactic strategies. Compositions containing antigens or polynucleotides encoding antigens of the invention provide effectively targeted vaccines for prevention and treatments of HSV infection.

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JUMBO APPLICATIONS / PATENTS

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IMMUNOLOGICALLY SIGNIFICANT HERPES SIMPLEX VIRUS
ANTIGENS AND METHODS FOR USING SAME

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TECHNICAL FIELD OF THE INVENTION

15 The invention relates to molecules, compositions and methods that can be used for the treatment and prevention of HSV infection. More particularly, the invention identifies epitopes of HSV proteins that can be used for methods, molecules and compositions having the antigenic specificity of HSV-specific T cells, and in particular, of CD8+ as well as CD4+ T cells.

BACKGROUND OF THE INVENTION

20 Cellular immune responses are required to limit the severity of recurrent HSV infection in humans. Initial genital HSV-2 infections are prolonged and severe, while recurrences are less severe and more frequently asymptomatic. Resolution of primary HSV-2 infection is associated with infiltration of antigen-specific T cells, including CD8+ cytotoxic T lymphocytes (CTLs). Serial lesion biopsy studies of recurrent HSV-2
25 infection in humans has shown a shift to CD8+ predominance as lesions mature and

correlation of local CTL activity with virus clearance (Koelle, DM et al., J. Clin. Invest. 1998, 101:1500-1508; Cunningham, AL et al., J. Clin. Invest. 1985, 75:226-233). Thus, HSV antigens recognized by CD8+ CTL can be used for novel therapies and vaccines.

5 The complete DNA sequence of herpes simplex virus (HSV) is approximately 150 kb and encodes about 85 known genes, each of which encodes a protein in the range of 50-1000 amino acids in length. Unknown are the immunogenic epitopes within these proteins, each epitope approximately 9-12 amino acids in length, that are capable of eliciting an effective T cell immune response to viral infection.

10 There remains a need to identify specific epitopes capable of eliciting an effective immune response to HSV infection. Such information can lead to the identification of more effective immunogenic antigens useful for the prevention and treatment of HSV infection.

SUMMARY OF THE INVENTION

15 The invention provides HSV antigens, polypeptides comprising HSV antigens, polynucleotides encoding the polypeptides, vectors, and recombinant viruses containing the polynucleotides, antigen-presenting cells (APCs) presenting the polypeptides, immune cells directed against HSV, and pharmaceutical compositions. The pharmaceutical compositions can be used both prophylactically and therapeutically. The antigens of the invention are recognized by T cells recovered from herpetic lesions. The invention additionally provides methods, including methods for preventing and treating HSV infection, for killing HSV-infected cells, for inhibiting viral replication, for enhancing secretion of antiviral and/or immunomodulatory lymphokines, and for enhancing production of HSV-specific antibody. For preventing and treating HSV infection, for enhancing secretion of antiviral and/or immunomodulatory lymphokines, 25 for enhancing production of HSV-specific antibody, and generally for stimulating and/or augmenting HSV-specific immunity, the method comprises administering to a subject a polypeptide, polynucleotide, recombinant virus, APC, immune cell or composition of the invention. The methods for killing HSV-infected cells and for inhibiting viral replication

comprise contacting an HSV-infected cell with an immune cell of the invention. The immune cell of the invention is one that has been stimulated by an antigen of the invention or by an APC that presents an antigen of the invention. A method for producing such immune cells is also provided by the invention. The method comprises
 5 contacting an immune cell with an APC, preferably a dendritic cell, that has been modified to present an antigen of the invention. In a preferred embodiment, the immune cell is a T cell such as a CD4⁺ or CD8⁺ T cell.

In one embodiment, the invention provides a composition comprising an HSV polypeptide. In one embodiment, the polypeptide comprises a U_L49 protein or a
 10 fragment thereof. In a preferred embodiment, the fragment of a U_L49 protein comprises amino acids 14-22, 21-35, 45-59, 49-57, 49-63, 105-190, 177-220 or 193-208 of U_L49 or a substitutional variant thereof. In another embodiment, the polypeptide comprises an ICP0 protein or a fragment thereof. In one embodiment, the fragment of an ICP0 protein comprises amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 or a
 15 substitutional variant thereof. In another embodiment, the polypeptide comprises a U_L48 protein or a fragment thereof. In one embodiment, the fragment of a U_L48 protein comprises amino acids 185-197, 209-221, 288-307 or 430-449 of VP16 (U_L48) or a substitutional variant thereof.

Also provided is an isolated polynucleotide that encodes a polypeptide of the invention,
 20 and a composition comprising the polynucleotide. The invention additionally provides a recombinant virus genetically modified to express a polynucleotide of the invention, and a composition comprising the recombinant virus. In preferred embodiments, the virus is a vaccinia virus, canary pox virus, HSV, lentivirus, retrovirus or adenovirus. A composition of the invention can be a pharmaceutical composition. The composition
 25 can optionally comprise a pharmaceutically acceptable carrier and/or an adjuvant.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic representing organization of the HSV genome in the region of 0.67-0.73 map units. Boundaries are approximate. HSV-1 X HSV-2 intertypic

recombinant viruses (IRV) are also shown. HSV-2 DNA is indicated by a solid line; HSV-1 DNA by a dashed line, and indeterminate regions by a multiple line. The HSV-2 *Bam*H I w fragment used for expression cloning is also shown.

Figure 1B is a bar graph showing proliferative responses of T-cell clones (TCC) to the indicated IRV. Data are delta CPM [^{3} H] thymidine incorporation compared to media alone, which was less than 500 cpm in each case.

Figure 2 is an immunoblot showing determination of the HSV viral phenotype of the UL49 gene product (VP22) of IRV DX32. Lysates of mock-infected cells and cells infected with the viral strains DX32, HSV-1 or HSV-2 were separated by SDS-PAGE, blotted, and probed with VP22-specific mAb. The molecular weights (kD) of marker proteins are shown at right.

Figure 3A is a bar graph showing T-cell proliferation elicited by various peptide epitopes in VP22 of HSV-2 using TCC 4.2E1. Antigen-presenting cells (APC) were autologous EBV-LCL. Antigens included β -galactosidase and fusion proteins used at 10 μ g/ml and peptides used at 3 μ M. Data are delta cpm [^{3} H] thymidine incorporation compared to media alone, which was less than 500 cpm in each case.

Figure 3B is a bar graph showing T-cell proliferation elicited by various peptide epitopes in VP22 of HSV-2 using TCC 1.L3D5.10.8. APC were autologous PBMC. Antigens included β -galactosidase and fusion proteins used at 10 μ g/ml and peptides used at 1 μ M. Data are delta cpm [^{3} H] thymidine incorporation compared to media alone, which was less than 500 cpm in each case.

Figure 3C is a bar graph showing T-cell proliferation elicited by various peptide epitopes in VP22 of HSV-2 using TCC ESL4.9. APC were autologous PBMC. Antigens included β -galactosidase and fusion proteins used at 10 μ g/ml and peptides used at 1 μ M. Data are delta cpm [^{3} H] thymidine incorporation compared to media alone, which was less than 500 cpm in each case.

Figure 4 shows secretion of IFN- γ by lesion-derived CD8⁺ T cell clones in response to COS-7 cells transfected with HLA class I heavy chain cDNA and infection with HSV-2 strain 333. Values are mean of duplicate IFN- γ secretion into medium, measured by ELISA.

- 5 Figure 5 shows secretion of IFN- γ by lesion-derived CD8⁺ T cell clone 5491.2000.48 in response to COS-7 cells co-transfected with HLA B*0702 cDNA and the indicated HSV-2 DNA fragments. All HSV-2 genes are full length except for U_L47, which is was tested in two segments encoding the indicated amino acids. Values are mean of duplicate IFN- γ secretion into the medium as measured by ELISA.
- 10 Figure 6 shows lysis by lesion-derived CD8 clones of autologous LCL loaded with HSV-2 peptides at the indicated concentrations. Data are percent specific ⁵¹Cr release at E:T 20:1. ●, Lysis by clone 5101.1999.23 of targets loaded with U_L47 551–559; ○, clone 1874.1991.22 and U_L47 289–298; ▼, clone 1874.1997.51 and ICP0 92–101; ▽, clone 5491.2000.48 and U_L49 49–57. Lysis of mock-loaded targets was <5% specific release for
- 15 each clone.

- Figure 7 shows cytolytic activity of CD8 CTL clones against cutaneous cells. *Top*, U_L49-specific clone 5491.2000.48, HLA B*0702-expressing fibroblasts from subject SJ, and peptide U_L49 aa 49–47. *Middle*, U_L 47-specific clone 1874.1991.22, HLA A*0201-bearing fibroblasts from subject 1874, and peptide U_L 47 aa 551–559. *Left*, Lysis of non-IFN- γ -
- 20 treated fibroblasts infected at the indicated MOI for 2 h before assay; *right*, fibroblasts pretreated for 3 days with 500 U/ml IFN- γ and then infected or treated with peptide. In each case, HLA-mismatched target fibroblasts had <5% specific release at E:T 2:1, 6:1, and 20:1. *Bottom*, HLA A*0201-bearing keratinocytes are used as target cells. *Left*, U_L 47-specific clone 5101.1999.23 and peptide U_L47 aa 289–298; *right*. U_L 47-specific clone
- 25 1874.1991.22 and peptide U_L 47 aa 551–559. HLA A*0201-bearing keratinocytes were mock treated or treated with 500 U/ml IFN- γ and then infected for 2 or 18 h with HSV-2 at MOI 25 or loaded with peptide for 90 min.

Figures 8A-E show the HLA types of the donors used in Example 5.

Figure 9 shows the CD8+ T cell peptide-screening hits.

Figure 10 shows the results of peptide screening for donor AD116.

Figure 11 shows the results of peptide screening for donors EB5491, TM10062 and HV5101.

- 5 Figure 12 shows results of peptide screening for donors AD104, AD116, AD120 and D477.

Figure 13 is a schematic representation of the positive genomic clone isolated from *Sau3A* I library of HSV-2 DNA (second line), which contained part of the ICP0 gene. The genomic clone was transfected into cells and primer A used for cDNA
10 synthesis. The exon-1/exon2 C-A (fifth line) and HLA B45 cDNAs stimulated interferon-gamma secretion from T cell clone (TCC) RW51 after transfection into Cos-7 cells. Exon-1 B-C cDNA (fourth line) was negative.

Figure 14 is a bar graph showing CTL activity of RW51 against vaccinia ICP0 and indicated concentrations of synthetic ICP0 92-105. Four-hour ⁵¹Cr release assay
15 with effector:target ratio 10:1. Spontaneous release all <20%.

Figure 15 is a graph showing CTL activity of RW51 against indicated concentrations of synthetic ICP0 92-101. Four-hour ⁵¹Cr release assay with effector:target ratio 10:1. Spontaneous release all <20%.

Figure 16 is a graph showing CTL activity of lymphocytes subject RW, derived from
20 peripheral blood and stimulated with a peptide of HSV-2 ICP0 amino acids 92-101. Four-hour ⁵¹Cr release assay with effector:target ratio of 10:1. Spontaneous release <20%. For each pair of bars, the upper bar represents data from a lesion-derived CD8 clone and the lower bar represents data from PBMC stimulated with peptide.

Figure 17 shows confirmation of HLA restricting allele, HSV-2 reactivity, and IFN-
25 gamma secretion by lesion CD8 clones.

Figure 18 shows peptide dose-response for lesion CD8 clone RW.1997.51 worked up by expression cloning.

Figure 19 is a line graph showing HLA restriction element for T-cell clone BM.17 response to peptide 437-449 of VP16 (UL48) of HSV-2. Proliferative responses are plotted versus concentration of viral peptide. Antigen presenting cells are EBV-LCL that are either autologous (closed circles), homozygous for HLA DQB1*0501 (open triangles), or homozygous for HLA DQB1*0201 (squares).

DETAILED DESCRIPTION OF THE INVENTION

The invention provides HSV antigens that are useful for the prevention and treatment of HSV infection. Disclosed herein are antigens and/or their constituent epitopes confirmed to be recognized by T-cells derived from herpetic lesions. In some embodiments, T-cells having specificity for antigens of the invention have demonstrated cytotoxic activity against virally infected cells. The identification of immunogenic antigens responsible for T-cell specificity facilitates the development of improved anti-viral therapeutic and prophylactic strategies. Compositions containing antigens or polynucleotides encoding antigens of the invention provide effectively targeted vaccines for prevention and treatment of HSV infection.

Definitions

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

As used herein, "polypeptide" includes proteins, fragments of proteins, and peptides, whether isolated from natural sources, produced by recombinant techniques or chemically synthesized. Polypeptides of the invention typically comprise at least about 6 amino acids, and preferably at least about 15 amino acids.

As used herein, "HSV polypeptide" includes HSV-1 and HSV-2, unless otherwise indicated. References to amino acids of HSV proteins or polypeptides are based on the genomic sequence information regarding HSV-2 as described in A. Dolan et al., 1998, J. Virol. 72(3):2010-2021. As noted below, the predicted polypeptide sequence of ICP0 of HSV-2 based on sequencing RNA from cells transfected with a fragment of ICP0 differs from the published sequence by the omission of amino acid Q26.

As used herein, "substitutional variant" refers to a molecule having one or more amino acid substitutions or deletions in the indicated amino acid sequence, yet retaining the ability to be specifically recognized by an immune cell. The amino acid sequence of a substitutional variant is preferably at least 80% identical to the native amino acid sequence, or more preferably, at least 90% identical to the native amino acid sequence. Typically, the substitution is a conservative substitution. One method for determining whether a molecule can be specifically recognized by an immune cell is the cytotoxicity assay described in D.M. Koelle et al., 1997, Human Immunol. 53:195-205. Other methods for determining whether a molecule can be specifically recognized by an immune cell are described in the examples provided hereinbelow, including the ability to stimulate secretion of interferon-gamma or the ability to lyse cells presenting the molecule. An immune cell will specifically recognize a molecule when, for example, stimulation with the molecule results in secretion of greater interferon-gamma than stimulation with control molecules. For example, the molecule may stimulate greater than 5 pg/ml, or preferably greater than 10 pg/ml, interferon-gamma secretion, whereas a control molecule will stimulate less than 5 pg/ml interferon-gamma.

As used herein, "vector" means a construct, which is capable of delivering, and preferably expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

As used herein, "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

- 5 The term "nucleic acid" or "polynucleotide" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogs of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides.

- 10 As used herein, "antigen-presenting cell" or "APC" means a cell capable of handling and presenting antigen to a lymphocyte. Examples of APCs include, but are not limited to, macrophages, Langerhans-dendritic cells, follicular dendritic cells, B cells, monocytes, fibroblasts and fibrocytes. Dendritic cells are a preferred type of antigen presenting cell. Dendritic cells are found in many non-lymphoid tissues but can migrate via the afferent lymph or the blood stream to the T-dependent areas of lymphoid organs. In non-lymphoid organs, dendritic cells include Langerhans cells and interstitial dendritic cells. In the lymph and blood, they include afferent lymph veiled cells and blood dendritic cells, respectively. In lymphoid organs, they include lymphoid dendritic cells and interdigitating cells.

- 20 As used herein, "modified" to present an epitope refers to antigen-presenting cells (APCs) that have been manipulated to present an epitope by natural or recombinant methods. For example, the APCs can be modified by exposure to the isolated antigen, alone or as part of a mixture, peptide loading, or by genetically modifying the APC to express a polypeptide that includes one or more epitopes.

- 25 As used herein, "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects. Examples of such salts include, but are not limited to, (a) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic

acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pantoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalenedisulfonic acids, polygalacturonic acid; (b) salts with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; or (c) salts formed with an organic cation formed from N,N'-dibenzylethylenediamine or ethylenediamine; or (d) combinations of (a) and (b) or (c), e.g., a zinc tannate salt; and the like. The preferred acid addition salts are the trifluoroacetate salt and the acetate salt.

10 As used herein, "pharmaceutically acceptable carrier" includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting
15 agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline.

Compositions comprising such carriers are formulated by well known conventional methods (see, for example, *Remington's Pharmaceutical Sciences*, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990).

20 As used herein, "adjuvant" includes those adjuvants commonly used in the art to facilitate the stimulation of an immune response. Examples of adjuvants include, but are not limited to, helper peptide; aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ);
25 AS-2 (Smith-Kline Beecham); QS-21 (Aquila); MPL or 3d-MPL (Corixa Corporation, Hamilton, MT); LEIF; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A;

muramyl tripeptide phosphatidyl ethanolamine or an immunostimulating complex, including cytokines (e.g., GM-CSF or interleukin-2, -7 or -12) and immunostimulatory DNA sequences. In some embodiments, such as with the use of a polynucleotide vaccine, an adjuvant such as a helper peptide or cytokine can be provided via a
5 polynucleotide encoding the adjuvant.

As used herein, "a" or "an" means at least one, unless clearly indicated otherwise.

As used herein, to "prevent" or "protect against" a condition or disease means to hinder, reduce or delay the onset or progression of the condition or disease.

HSV Polypeptides

10 In one embodiment, the invention provides an isolated herpes simplex virus (HSV) polypeptide. The polypeptide comprises an ICP0, VP16 (UL48), or UL49 protein or a fragment thereof. In one embodiment, the fragment comprises amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 or a substitutional variant thereof. In other
15 embodiments, the fragment comprises amino acids 185-197, 209-221, 288-307, 430-449 or 437-449 of VP16 (UL48) or a substitutional variant thereof. In another embodiment, the fragment comprises amino acids 14-22, 21-35, 45-59, 47-55, 49-57, 49-63, 105-190, 177-220 or 193-208 of UL49 or a substitutional variant thereof. The reference to amino acid residues is made with respect to the proteins of the HSV-2 genome as described in A. Dolan et al., 1998, J. Virol. 72(3):2010-2021. The amino acid sequences of ICP0,
20 VP16 (UL48), and UL49 are as follows.

ICP0 amino acid sequence (SEQ ID NO: 1)

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1  meprpgtssr adpgperppr qtpgtqpaap hawgmlndmq wlassdseee tevgisdddl
61  hrdstseags tdtemfeagl mdaatpparp paerqgsptp adaqgscggg pvgeeeaeag
25 121  gggdvcavct deiapplrcq sfpclhpfci pcmktwiplr ntcplcntpv aylivgvtas
181  gsfstipivn dprtrveaea avragtavdf iwtgnprtap rslslgghtv ralsptppwp
241  gtddedddla dvdyvppapr raprrgggga gatrgtsqpa atrpappgap rssssggapl
301  ragvgsgsgg gpavaavvpr vaslppaagg graqarrvge daaaaegrtp parqpaaqe
361  ppivisdspg psprrpagpg plsfvssssa qvssgpgggg lpqssgraar praavaprvr
30 421  sppraaaapv vsasadaagp appavpvdah raprsrmtqa qtdtqaqslg ragatdargs
481  gpggaeggpg vprgtntpga aphaaegaaa rprkrrgsds gpaasssasa saaprsplap
541  qvgakraap rrapdsdsgd rghgplapas agaappsasp ssqaavaaas sssassssas
601  sssassssas sssassssas sssasssagg aggsvasasg agerretslg praaaprgpr
661  kcarktrhae ggpepgardp apgltrylpi agvssvvala pyvnktvtgd clpvldmetg

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721 higayvvlvd qtgnvadllr aaapawsrrt llpeharnvcv rppdyptppa sewnslwmt
 781 vgnmlfdqgt lvgaldfhgl rsrhpwsreq gapapagdap aghge

VP16 (UL48) amino acid sequence (SEQ ID NO: 2)

5 1 mdllvddlfa dadgvspppp rpaggpkntp aapplyatgr lsqaqlmpsp pmpvppaalf
 61 nrllddlgfs agpalctmld twnedlfsgf ptnadmyrec kflstlpsdv idwgdahvpe
 121 rspidirahg dvaftlpat rdelpsyysa magffrgelr areesyrtvl anfcslalyry
 181 lrasvrqlhr qahmrgrnrd lremlrttia dryyretarl arvlflhlyl flsreilwaa
 241 yaeqmmrpd1 fdglccdlles wrqlaclfqp lmfingsltv rgvpvearrl relnhirehl
 10 301 nlplvrsaaa eepgaplttp pvlqgnqars sgyfmlira kldsyssvat segesvmreh
 361 aysrgrtrnn ygstieglld lpddddapae aglvaprmsf lsagqrprrl sttapitdvs
 421 lgdelrldge evdmtpadal ddfdlemlgd vespspgmth dpvsygaldiv ddfefeqmft
 481 damgiddfgg

15 UL 49 amino acid sequence (SEQ ID NO: 3)

1 mtsrrsvksc preaprgthe elyygpvspa dpesprddfr rgagpmrarp rgevrfllhyd
 61 eagyalrds ssdddesrdt arprrsasva gshgpgpara ppppggpvga ggrshappar
 121 tpkmtrgapk asatpatdpa rgrpaqads avlldapapt asgrtktpaq glakklhfst
 181 appsptapwt prvagfnkrv fcaavgrlaa tharlaavql wdmsrphtde dlneildltt
 20 241 irvtvcegkn llqranelvn pdaaqdvdat aaargrpagr aaatarapar sasrprple

The polypeptide can be a fusion protein. In one embodiment, the fusion protein is soluble. A soluble fusion protein of the invention can be suitable for injection into a subject and for eliciting an immune response. Within certain embodiments, a
 25 polypeptide can be a fusion protein that comprises multiple polypeptides as described herein, or that comprises at least one polypeptide as described herein and an unrelated sequence. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the
 30 native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the protein or to enable the protein to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the protein.

35 Fusion proteins may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion protein is expressed as a recombinant protein, allowing the production of increased levels, relative to a non-fused protein, in an expression

system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion protein that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., 1985, Gene 40:39-46; Murphy et al., 1986, Proc. Natl. Acad. Sci. USA 83:8258-8262; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are present 3' to the DNA sequence encoding the second polypeptide.

Fusion proteins are also provided that comprise a polypeptide of the present invention together with an unrelated immunogenic protein. Preferably the immunogenic protein is capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (see, for example, Stoute et al., 1997, New Engl. J. Med., 336:86-9).

Within preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium *Haemophilus influenza B* (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (e.g., the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenza virus, NS1 (hemagglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the *LytA* gene; Gene 43:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (see Biotechnology 10:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion protein. A

repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

In some embodiments, it may be desirable to couple a therapeutic agent and a polypeptide of the invention, or to couple more than one polypeptide of the invention.

5 For example, more than one agent or polypeptide may be coupled directly to a first polypeptide of the invention, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used. Some molecules are particularly suitable for intercellular trafficking and protein delivery, including, but not limited to, VP22 (Elliott and O'Hare, 1997, *Cell* 88:223-233; see also Kim et al., 1997, *J. Immunol.* 159:1666-1668; 10 Rojas et al., 1998, *Nature Biotechnology* 16:370; Kato et al., 1998, *FEBS Lett.* 427(2):203-208; Vives et al., 1997, *J. Biol. Chem.* 272(25):16010-7; Nagahara et al., 1998, *Nature Med.* 4(12):1449-1452).

A carrier may bear the agents or polypeptides in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as 15 albumins (e.g., U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (e.g., U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patent Nos. 4,429,008 and 4,873,088).

In general, polypeptides (including fusion proteins) and polynucleotides as described 20 herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered 25 to be isolated if, for example, it is cloned into a vector that is not part of the natural environment.

The polypeptide can be isolated from its naturally occurring form, produced by recombinant means or synthesized chemically. Recombinant polypeptides encoded by

DNA sequences described herein can be readily prepared from the DNA sequences using any of a variety of expression vectors known to those of ordinary skill in the art. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a
5 recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably the host cells employed are *E. coli*, yeast or a mammalian cell line such as Cos or CHO. Supernatants from the soluble host/vector systems that secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be
10 applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide.

Fragments and other variants having less than about 100 amino acids, and generally less than about 50 amino acids, may also be generated by synthetic means, using techniques
15 well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, wherein amino acids are sequentially added to a growing amino acid chain (Merrifield, 1963, J. Am. Chem. Soc. 85:2146-2149). Equipment for automated synthesis of polypeptides is commercially available from
20 suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

Variants of the polypeptide for use in accordance with the invention can have one or more amino acid substitutions, deletions, additions and/or insertions in the amino acid sequence indicated that result in a polypeptide that retains the ability to elicit an immune
25 response to HSV or HSV-infected cells. Such variants may generally be identified by modifying one of the polypeptide sequences described herein and evaluating the reactivity of the modified polypeptide using a known assay such as a T cell assay described herein. Polypeptide variants preferably exhibit at least about 70%, more preferably at least about 90%, and most preferably at least about 95% identity to the

identified polypeptides. These amino acid substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative".

A "conservative" substitution is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

One can readily confirm the suitability of a particular variant by assaying the ability of the variant polypeptide to elicit an immune response. The ability of the variant to elicit an immune response can be compared to the response elicited by the parent polypeptide assayed under identical circumstances. One example of an immune response is a cellular immune response. The assaying can comprise performing an assay that measures T cell stimulation or activation. Examples of T cells include CD4 and CD8 T cells.

One example of a T cell stimulation assay is a cytotoxicity assay, such as that described in Koelle, DM et al., Human Immunol. 1997, 53;195-205. In one example, the cytotoxicity

assay comprises contacting a cell that presents the antigenic viral peptide in the context of the appropriate HLA molecule with a T cell, and detecting the ability of the T cell to kill the antigen presenting cell. Cell killing can be detected by measuring the release of radioactive ^{51}Cr from the antigen presenting cell. Release of ^{51}Cr into the medium from the antigen presenting cell is indicative of cell killing. An exemplary criterion for increased killing is a statistically significant increase in counts per minute (cpm) based on counting of ^{51}Cr radiation in media collected from antigen presenting cells admixed with T cells as compared to control media collected from antigen presenting cells admixed with media.

10 Polynucleotides, Vectors, Host Cells and Recombinant Viruses

The invention provides polynucleotides that encode one or more polypeptides of the invention. The complete genome sequence of HSV-2, strain HG52, can be found on the NCBI web site (www.ncbi.nih.gov), Accession No. Z86099. The polynucleotide can be included in a vector. The vector can further comprise an expression control sequence operably linked to the polynucleotide of the invention. In some embodiments, the vector includes one or more polynucleotides encoding other molecules of interest. In one embodiment, the polynucleotide of the invention and an additional polynucleotide can be linked so as to encode a fusion protein.

Within certain embodiments, polynucleotides may be formulated so to permit entry into a cell of a mammal, and expression therein. Such formulations are particularly useful for therapeutic purposes, as described below. Those of ordinary skill in the art will appreciate that there are many ways to achieve expression of a polynucleotide in a target cell, and any suitable method may be employed. For example, a polynucleotide may be incorporated into a viral vector such as, but not limited to, adenovirus, adeno-associated virus, retrovirus, or vaccinia or other pox virus (e.g., avian pox virus). Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or selection of transduced cells) and/or a targeting moiety,

such as a gene that encodes a ligand for a receptor on a specific target cell, to render the vector target specific. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art.

5 The invention also provides a host cell transformed with a vector of the invention. The transformed host cell can be used in a method of producing a polypeptide of the invention. The method comprises culturing the host cell and recovering the polypeptide so produced. The recovered polypeptide can be purified from culture supernatant.

10 Vectors of the invention can be used to genetically modify a cell, either *in vivo*, *ex vivo* or *in vitro*. Several ways of genetically modifying cells are known, including transduction or infection with a viral vector either directly or via a retroviral producer cell, calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes or microspheres containing the DNA, DEAE dextran, receptor-mediated endocytosis, electroporation, micro-injection, and many other techniques known to those of skill. See, e.g., Sambrook et al. Molecular Cloning - A Laboratory Manual (2nd ed.) 1-3, 1989; and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement).

20 Examples of viral vectors include, but are not limited to retroviral vectors based on, e.g., HIV, SIV, and murine retroviruses, gibbon ape leukemia virus and other viruses such as adeno-associated viruses (AAVs) and adenoviruses. (Miller et al. 1990, Mol. Cell Biol. 10:4239; J. Kolberg 1992, NIH Res. 4:43, and Cornetta et al. 1991, Hum. Gene Ther. 2:215). Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), ecotropic retroviruses, simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations. 25 See, e.g. Buchscher et al. 1992, J. Virol. 66(5):2731-2739; Johann et al. 1992, J. Virol. 66(5):1635-1640; Sommerfelt et al. 1990, Virol. 176:58-59; Wilson et al. 1989, J. Virol. 63:2374-2378; Miller et al. 1991, J. Virol. 65:2220-2224, and Rosenberg and Fauci 1993 in Fundamental Immunology, Third Edition, W.E. Paul (ed.) Raven Press, Ltd., New York

and the references therein; Miller et al. 1990, Mol. Cell Biol. 10:4239; R. Kolberg 1992, J. NIH Res. 4:43; and Cornetta et al. 1991, Hum. Gene Ther. 2:215.

In vitro amplification techniques suitable for amplifying sequences to be subcloned into an expression vector are known. Examples of such *in vitro* amplification methods, including the polymerase chain reaction (PCR), ligase chain reaction (LCR), Q β -replicase amplification and other RNA polymerase mediated techniques (e.g., NASBA), are found in Sambrook et al. 1989, Molecular Cloning - A Laboratory Manual (2nd Ed) 1-3; and U.S. Patent No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, CA 1990. Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Patent No. 5,426,039.

The invention additionally provides a recombinant microorganism genetically modified to express a polynucleotide of the invention. The recombinant microorganism can be useful as a vaccine, and can be prepared using techniques known in the art for the preparation of live attenuated vaccines. Examples of microorganisms for use as live vaccines include, but are not limited to, viruses and bacteria. In a preferred embodiment, the recombinant microorganism is a virus. Examples of suitable viruses include, but are not limited to, vaccinia virus, canary pox virus, retrovirus, lentivirus, HSV and adenovirus.

Compositions

The invention provides compositions that are useful for treating and preventing HSV infection. The compositions can be used to inhibit viral replication and to kill virally-infected cells. In one embodiment, the composition is a pharmaceutical composition. The composition can comprise a therapeutically or prophylactically effective amount of a polypeptide, polynucleotide, recombinant virus, APC or immune cell of the invention. An effective amount is an amount sufficient to elicit or augment an immune response, e.g., by activating T cells. One measure of the activation of T cells is a cytotoxicity assay, as described in D.M. Koelle et al., 1997, Human Immunol. 53:195-205. In some embodiments, the composition is a vaccine.

The composition can optionally include a carrier, such as a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention. Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, and carriers include aqueous isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, preservatives, liposomes, microspheres and emulsions.

The composition of the invention can further comprise one or more adjuvants. Examples of adjuvants include, but are not limited to, helper peptide, alum, Freund's, muramyl tripeptide phosphatidyl ethanolamine or an immunostimulating complex, including cytokines. In some embodiments, such as with the use of a polynucleotide vaccine, an adjuvant such as a helper peptide or cytokine can be provided via a polynucleotide encoding the adjuvant. Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other viral antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides of the invention, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art,

such as those described by Rolland, 1998, Crit. Rev. Therap. Drug Carrier Systems 15:143-198, and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of
5 a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for
10 example, in Fisher-Hoch et al., 1989, Proc. Natl. Acad. Sci. USA 86:317-321; Flexner et al., 1989, Ann. My Acad. Sci. 569:86-103; Flexner et al., 1990, Vaccine 8:17-21; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91102805; Berkner, 1988, Biotechniques 6:616-627; Rosenfeld et al., 1991, Science 252:431-434; Kolls et al., 1994, Proc. Natl.
15 Acad. Sci. USA 91:215-219; Kass-Eisler et al., 1993, Proc. Natl. Acad. Sci. USA 90:11498-11502; Guzman et al., 1993, Circulation 88:2838-2848; and Guzman et al., 1993, Cir. Res. 73:1202-1207. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., 1993, Science 259:1745-1749 and
20 reviewed by Cohen, 1993, Science 259:1691-1692. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending
25 on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral
30 administration, any of the above carriers or a solid carrier, such as mannitol, lactose,

starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S.

5 Patent Nos. 4,897,268 and 5,075,109.

Such compositions may also comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and/or preservatives.

10 Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of adjuvants may be employed in the vaccines of this invention. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable
15 adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated
20 tyrosine acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Within the vaccines provided herein, the adjuvant composition is preferably designed to induce an immune response predominantly of the Th1 type. High levels of Th1-type
25 cytokines (e.g., IFN- γ , IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (e.g., IL-4, IL-5, IL-6, IL-10 and TNF- β) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a

patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays.

5 For a review of the families of cytokines, see Mosmann and Coffman, 1989, Ann. Rev. Immunol. 7:145-173.

Preferred adjuvants for use in eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPLTM adjuvants

10 are available from Corixa Corporation (see US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555.

Another preferred adjuvant is a saponin, preferably QS21, which may be used alone or in

15 combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprises an oil-in-water emulsion and

20 tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210. Another adjuvant that may be used is AS-2 (Smith-Kline Beecham). Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immune response enhancer and a suitable carrier or excipient.

25 The compositions described herein may be administered as part of a sustained release formulation (i.e., a formulation such as a capsule or sponge that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations

may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component
 5 release. The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune
 10 response that targets HSV-infected cells. Delivery vehicles include antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have antiviral effects *per se* and/or to be
 15 immunologically compatible with the receiver (i.e., matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or
 20 progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, Nature 392:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic immunity (see Timmerman and Levy, Ann. Rev. Med. 50:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic
 25 processes (dendrites) visible *in vitro*) and based on the lack of differentiation markers of B cells (CD19 and CD20), T cells (CD3), monocytes (CD14) and natural killer cells (CD56), as determined using standard assays. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are

contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (Zitvogel et al., 1998, Nature Med. 4:594-600).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF α to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF α , CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well-characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fc γ receptor, mannose receptor and DEC-205 marker. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (e.g., CD54 and CD11) and costimulatory molecules (e.g., CD40, CD80 and CD86). APCs may generally be transfected with a polynucleotide encoding a polypeptide (or portion or other variant thereof) such that the polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the

art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., 1997, Immunology and Cell Biology 75:456-460. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the tumor polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (e.g., vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (e.g., a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

10 Administration of the Compositions

Treatment includes prophylaxis and therapy. Prophylaxis or treatment can be accomplished by a single direct injection at a single time point or multiple time points. Administration can also be nearly simultaneous to multiple sites. Patients or subjects include mammals, such as human, bovine, equine, canine, feline, porcine, and ovine animals. Preferably, the patients or subjects are human.

Compositions are typically administered *in vivo* via parenteral (e.g. intravenous, subcutaneous, and intramuscular) or other traditional direct routes, such as buccal/sublingual, rectal, oral, nasal, topical, (such as transdermal and ophthalmic), vaginal, pulmonary, intraarterial, intraperitoneal, intraocular, or intranasal routes or directly into a specific tissue.

The compositions are administered in any suitable manner, often with pharmaceutically acceptable carriers. Suitable methods of administering cells in the context of the present invention to a patient are available, and, although more than one route can be used to administer a particular cell composition, a particular route can often provide a more immediate and more effective reaction than another route.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time, or to inhibit

infection or disease due to infection. Thus, the composition is administered to a patient in an amount sufficient to elicit an effective immune response to the specific antigens and/or to alleviate, reduce, cure or at least partially arrest symptoms and/or complications from the disease or infection. An amount adequate to accomplish this is
5 defined as a "therapeutically effective dose."

The dose will be determined by the activity of the composition produced and the condition of the patient, as well as the body weight or surface areas of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side effects that accompany the administration of a particular composition
10 in a particular patient. In determining the effective amount of the composition to be administered in the treatment or prophylaxis of diseases such as HSV infection, the physician needs to evaluate the production of an immune response against the virus, progression of the disease, and any treatment-related toxicity.

For example, a vaccine or other composition containing a subunit HSV protein can
15 include 1-10,000 micrograms of HSV protein per dose. In a preferred embodiment, 10-1000 micrograms of HSV protein is included in each dose in a more preferred embodiment 10-100 micrograms of HSV protein dose. Preferably, a dosage is selected such that a single dose will suffice or, alternatively, several doses are administered over the course of several months. For compositions containing HSV polynucleotides or
20 peptides, similar quantities are administered per dose.

In one embodiment, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when
25 administered as described above, is capable of promoting an antiviral immune response, and is at least 10-50% above the basal (i.e., untreated) level. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome in vaccinated patients as compared to non-vaccinated patients. In general, for

pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 0.1 μ g to about 5 mg per kg of host. Preferably, the amount ranges from about 10 to about 1000 μ g per dose. Suitable volumes for administration will vary with the size, age and immune status of the patient, but will typically range from about 0.1 mL to about 5 mL, with volumes less than about 1 mL being most common.

Compositions comprising immune cells are preferably prepared from immune cells obtained from the subject to whom the composition will be administered. Alternatively, the immune cells can be prepared from an HLA-compatible donor. The immune cells are obtained from the subject or donor using conventional techniques known in the art, exposed to APCs modified to present an epitope of the invention, expanded *ex vivo*, and administered to the subject. Protocols for *ex vivo* therapy are described in Rosenberg et al., 1990, New England J. Med. 9:570-578. In addition, compositions can comprise APCs modified to present an epitope of the invention.

Immune cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions typically use intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive polypeptides as provided herein may be used to enrich and rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage, monocyte, fibroblast and/or B cells, may be pulsed with immunoreactive polypeptides or transfected with one or more polynucleotides using standard techniques well known in the art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*. Studies have shown that cultured effector cells can be induced

to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (see, for example, Cheever et al., 1997, Immunological Reviews 157:177).

Administration by many of the routes of administration described herein or otherwise known in the art may be accomplished simply by direct administration using a needle, catheter or related device, at a single time point or at multiple time points.

In Vivo Testing of Identified Antigens

Conventional techniques can be used to confirm the *in vivo* efficacy of the identified HSV antigens. For example, one technique makes use of a mouse challenge model. Those skilled in the art, however, will appreciate that these methods are routine, and that other models can be used.

Once a compound or composition to be tested has been prepared, the mouse or other subject is immunized with a series of injections. For example up to 10 injections can be administered over the course of several months, typically with one to 4 weeks elapsing between doses. Following the last injection of the series, the subject is challenged with a dose of virus established to be a uniformly lethal dose. A control group receives placebo, while the experimental group is actively vaccinated. Alternatively, a study can be designed using sublethal doses. Optionally, a dose-response study can be included. The end points to be measured in this study include death and severe neurological impairment, as evidenced, for example, by spinal cord gait. Survivors can also be sacrificed for quantitative viral cultures of key organs including spinal cord, brain, and the site of injection. The quantity of virus present in ground up tissue samples can be measured. Compositions can also be tested in previously infected animals for reduction in recurrence to confirm efficacy as a therapeutic vaccine.

Efficacy can be determined by calculating the IC₅₀, which indicates the micrograms of vaccine per kilogram body weight required for protection of 50% of subjects from death. The IC₅₀ will depend on the challenge dose employed. In addition, one can calculate the

LD₅₀, indicating how many infectious units are required to kill one half of the subjects receiving a particular dose of vaccine. Determination of the post mortem viral titer provides confirmation that viral replication was limited by the immune system.

A subsequent stage of testing would be a vaginal inoculation challenge. For acute protection studies, mice can be used. Because they can be studied for both acute protection and prevention of recurrence, guinea pigs provide a more physiologically relevant subject for extrapolation to humans. In this type of challenge, a non-lethal dose is administered, the guinea pig subjects develop lesions that heal and recur. Measures can include both acute disease amelioration and recurrence of lesions. The intervention with vaccine or other composition can be provided before or after the inoculation, depending on whether one wishes to study prevention versus therapy.

Methods

The invention provides a method for treatment and/or prevention of HSV infection in a subject. The method comprises administering to the subject a composition of the invention. The composition can be used as a therapeutic or prophylactic vaccine. In one embodiment, the HSV is HSV-2. Alternatively, the HSV is HSV-1. The invention additionally provides a method for inhibiting HSV replication, for killing HSV-infected cells, for increasing secretion of lymphokines having antiviral and/or immunomodulatory activity, and for enhancing production of herpes-specific antibodies. The method comprises contacting an HSV-infected cell with an immune cell directed against an antigen of the invention, for example, as described in the Examples presented herein. The contacting can be performed *in vitro* or *in vivo*. In a preferred embodiment, the immune cell is a T cell. T cells include CD4 and CD8 T cells. Compositions of the invention can also be used as a tolerizing agent against immunopathologic disease.

In addition, the invention provides a method of producing immune cells directed against HSV. The method comprises contacting an immune cell with an HSV polypeptide of the invention. The immune cell can be contacted with the polypeptide via an antigen-presenting cell, wherein the antigen-presenting cell is modified to present an antigen

- included in a polypeptide of the invention. Preferably, the antigen-presenting cell is a dendritic cell. The cell can be modified by, for example, peptide loading or genetic modification with a nucleic acid sequence encoding the polypeptide. In one embodiment, the immune cell is a T cell. T cells include CD4 and CD8 T cells. Also provided are
- 5 immune cells produced by the method. The immune cells can be used to inhibit HSV replication, to kill HSV-infected cells, *in vitro* or *in vivo*, to increase secretion of lymphokines having antiviral and/or immunomodulatory activity, to enhance production of herpes-specific antibodies, or in the treatment or prevention of HSV infection in a subject.
- 10 The invention also provides a diagnostic assay. The diagnostic assay can be used to identify the immunological responsiveness of a patient suspected of having a herpetic infection and to predict responsiveness of a subject to a particular course of therapy. The assay comprises exposing T cells of a subject to an antigen of the invention, in the context of an appropriate APC, and testing for immunoreactivity by, for example,
- 15 measuring IFN γ , proliferation or cytotoxicity. Suitable assays are described in more detail in the Examples.

EXAMPLES

- The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any
- 20 way to otherwise limit the scope of the invention.

Example 1: Identification of Viral Epitopes in HSV-2 Tegument Proteins

- This example shows the use of expression cloning with full-length viral DNA to identify T-cell antigens. Described herein are HSV epitopes recognized by lesion-infiltrating T-cells discovered by expression cloning. Details of the methods are described in U.S.
- 25 Patent No. 6,375,952, issued April 23, 2002.

Lymphocyte Functional Assays

Triplicate proliferation assay wells contained 10^4 cloned T-cells, 10^5 irradiated (3300 rad) PBMC or 2.5×10^4 irradiated (8000 rad) EBV-LCL as antigen presenting cells (APC), and antigen in 200 μ l T-cell media (D.M. Koelle et al., 1997, Human. Immunol., 53:195-205) in 96-well U-bottom plates. When heat-killed bacteria were used as antigen, the equivalent of 10^5 cfu/well (prior to inactivation) was added and gentamicin (20 μ g/ml) was included. After 72 hours, 1 μ Ci/well [^{3} H] thymidine was added for 18 hours, cells were harvested, and incorporation of thymidine evaluated by liquid scintillation counting. Standard deviations were less than 10% of the mean values. Results are reported as mean cpm or as delta cpm = mean cpm for experimental antigen minus mean cpm for control antigen. Control antigen was mock-infected cell lysate for whole viral antigens and pUEX2-derived β -galactosidase for recombinant protein preparations. To determine the reactivity of bulk-cultured lesion-derived T-cells, fusion proteins or control β -galactosidase were used at 10 μ g/ml. To determine HLA restricting loci, HLA DR-specific mAb L243 (V.G. Preston et al., 1978, J. Virol., 28:499-517), HLA DP-specific mAb B7.21 (A.J. Watson et al., 1983, Nature, 304:358-360), or HLA DQ-specific mAb SpV-L3 (H. Spits et al., 1984, Eur. J. Immunol., 14:299-304) were used as described (D.M. Koelle et al., 1994, J. Virol. 68:2803-2810).

Cytolysis assays were performed in triplicate using 4-hour [^{51}]Cr release as described (D.M. Koelle et al., 1993, J. Clin. Invest., 91:961-968). Target EBV-LCL were infected for 18 hours with HSV-3 at a multiplicity of infection of 30 or pulsed with 1.0 μ M peptide for 90 minutes prior to washing as described (W.W. Kwok et al., 1996, J. Exp. Med., 183:1253-1258). The effector to target ratio was 20:1. Spontaneous release was less than 28%.

Results

Fine Localization of T-cell Epitopes

To reduce the complexity of libraries for expression cloning, TCC recognizing antigen(s) partially mapped using HSV-1 X HSV-2 intertypic recombinant viruses (IRV) were
 5 selected. HSV DNA near 0.7 map units encodes T-cell antigens in addition to VP16. Epitope mapping for TCC 4.2E1 and 2.3 (D.M. Koelle et al., 1994, J. Virol., 68:2803-2810) was improved with IRV DX32 (Figure 1A). This HSV-2 based virus contains a block of HSV-1 DNA near 0.7 map units (V.G. Preston et al., 1978, J. Virol., 28:499-517). The U_L48 gene product has the HSV-2 phenotype, as shown by reactivity with
 10 HSV-2 type-specific, VP16-specific (D.M. Koelle et al., 1994, J. Virol., 68:2803-2810) T-cell clone 1A.B.25. The U_L49 (Figure 2) and U_L50 gene products (M.V. Williams, 1987, Virology, 156:282-292; F. Wohlrab, 1982, J. Virol., 43:935-942) also have a HSV-2 phenotype. The HSV-2 DNA present in IRV DX32 therefore includes U_L48, U_L49, U_L50, and most likely the intervening U_L49.5. Since TCC 4.2E1 and 2.3 react with
 15 RS1G31 and DX32, but not with RP2 (Figure 1B), recognition of U_L49, U_L49.5, or U_L50 is most likely.

Expression cloning to determine T-cell antigens

The *Bam*HI w fragment of HSV-2 was selected for expression cloning, since it contains the U_L49, U_L49.5, and most of the U_L50 coding sequences (A. Cress and S.J.
 20 Triezenberg, 1991, Gene, 103:235-238; G.D. Elliott and D.M. Meredith, 1992, J. Gen. Virol., 73:723-736; N.J. Maitland et al., 1982, Infect. Immun., 38:834-842). 70-90% of random colonies contained an insert; all were of viral origin. The most active libraries (Table 1) for each TCC (pUEX1 for TCC 4.2E1, pUEX 3 for TCC 2.3) were selected and an individual reactive bacterial clone detected by sequential testing of pools and
 25 individual colonies (Table 2). Clone 1.1.3 encodes a fusion protein eliciting proliferation by TCC 4.2E1. This clone contains a backwards 80 bp *Sma* I fragment of U_L49, a 262 bp *Sma* I fragment of HSV-2 U_L49 DNA predicted to encode amino acids 105 to 190,

forward and in-frame with regards to β -galactosidase, and a 246 bp *Sma* I fragment of U_L49 forward but out of frame due to a deletion of a single C residue at the 262 bp *Sma* I fragment-242 bp *Sma* I fragment junction. Clone 3.19 contained a 583 bp *Sma* I fragment encoding amino acids 118-312 of U_L50, followed by backwards 80 and 96 bp *Sma* I fragments of U_L49.

Table 1. Identification of protein libraries eliciting proliferation (mean cpm [³H]thymidine incorporation) of HSV-specific TCC. Autologous EBV-LCL (clones 4.2E1 and 2.3) or PBMC were used as APC and library-derived fusion protein antigens were diluted 1:300. Data are mean cpm [³H] thymidine incorporation.

TCC	library ¹			control stimuli ²	
	pUEX1- <i>Bam</i> HI "w"- <i>Sma</i> I	pUEX2- <i>Bam</i> HI "w"- <i>Sma</i> I	pUEX3- <i>Bam</i> H I "w"- <i>Sma</i> I	media	HSV-2
4.2E1	10,105	4,150	1,903	286	21,591
2.3	418	785	2,279	102	11,014
	pUEX1-HG52- <i>Sma</i> I- <i>Alu</i> I	pUEX2-HG52- <i>Sma</i> I- <i>Alu</i> I	pUEX3- HG52- <i>Sma</i> I- <i>Alu</i> I		
ESL4.9	-52	-25	16,235	146	66,013
ESL2.20	1	768	5,427	123	13,359

¹ Library names list expression vector, name of HSV-2 restriction fragment or strain of full-length viral DNA, and restriction enzyme(s) used to digest viral DNA.

² 10⁵ autologous irradiated (3300 rad) PBMC and either mock-infected cell lysate or UV-treated HSV-2 antigen.

Identification of T-cell antigens was confirmed by targeted subcloning and overlapping peptides. The 262 bp *Sma* I fragment of U_L49 of HSV-2 encoding amino acids 105-190 was subcloned into pUEX3 to yield plasmid 49.262.12. This protein stimulated TCC 4.2E1 (Table 2). Only peptide 105-126 of VP22 of HSV-2

(GGPVGAGGRSHAPPARTPKMTR; SEQ ID NO: 4) was stimulatory (Figure 3). DNA fragments encoding UL50 118-312 and 118-250 were subcloned into pUEX3. Fusion proteins expressing these fragments were active (Table 2).

Table 2. Antigenic specificity of HSV-2 reactive TCC. Bacterially-derived recombinant fusion protein antigens were used at 1:900 dilution. Autologous EBV-LCL (clone 4.2E1) or PBMC were used as APC. Data are delta cpm [³H] thymidine incorporation compared to media, which was less than 500 cpm in each case.

<u>TCC</u>	<u>Clone name</u>	<u>recombinant antigen</u>		<u>control antigens</u>		
		<u>viral sequence</u> ¹	<u>cpm</u>	<u>pUEX2 β-gal</u>	<u>HSV-1</u>	<u>HSV-2</u>
4.2E1	1.1.3	VP22 105-190	4,875	93	nd	nd
	49.262.1	VP22 105-190	6,898			
	2 ²					
2.3	3.19	UL50 118-312	43,971	231	543	53,032
	50.583.4	UL50 118-312	34,453			
	4 ³					
	50.397 ³	UL50 118-250	66,501			
ESL4.9	C11	VP22 177-220	59,400	166	112,803	64,685

¹ Amino acids predicted forward and in-frame with β-galactosidase from sequence data.

² Confirmatory subclone of 1.1.3 containing only a 262 bp *Sma* I fragment of UL49 DNA forward and in-frame with pUEX3.

³ Confirmatory subclones of 3.19 containing a 583 bp *Sma* I fragment of UL50 or a 397 bp *Sma* I-*Stu* I fragment of UL50 DNA forward and in-frame with pUEX3.

Evaluation of random colonies from full-length HSV-2 DNA libraries showed that 80-100% contained plasmids with an insert; 80-100% of inserts were of viral origin. For

both TCC ESL4.9 and ESL2.20, only the pUEX3 protein library elicited lymphoproliferation (Table 1). Since the libraries were more complex than for those made from the *Bam*HI w fragment, 2,000-3,000 bacterial transformants were screened by a combinatorial method. In preliminary experiments, heat-killed, washed bacteria
 5 were found to substitute for inclusion body preparations of protein in lymphoproliferation assays at the pool (5-12 bacterial clones) and final assay stages.

Sequencing of plasmids in positive bacteria showed that TCC ESL4.9 recognized a 44 amino acid fragment of U_L49 gene product VP22 (amino acids 177-220), while TCC ESL2.20 recognized a 34 amino acid fragment of U_L21 (amino acids 148-181) (Table 2).
 10 In both cases single *Alu* I fragments of HSV-2 DNA were inserted in-frame and forwards. Peptide mapping revealed that amino acids 187-206 (Figure 3C) stimulated TCC ESL4.9.

Fusion proteins as probes of bulk lesion-infiltrating T-cells

Newly discovered T-cell antigens were added to the panel of HSV antigens used to probe
 15 bulk cultures of lesion-infiltrating T-cells. The first available specimens were a set of four biopsies (2 mm each) obtained from day 5 (virus culture positive) of a buttock recurrence of HSV-2 from patient 1 (D.M. Koelle et al., 1998, J. Clin. Invest. 101:1500-09; D.M. Koelle et al., 1994, J. Virol., 68:2803-2810). All four biopsies showed reactivity with VP22 105-190 but not β -galactosidase, glycoproteins B or D, or VP16. TCC were
 20 derived after restimulating the original bulk culture for one cycle with VP22 105-190 fusion protein. Proliferative responses of TCC 1.L3D5.10.8 (Figure 3B) to VP22 (105-190) and constituent peptides document a third T-cell epitope in VP22 contained within amino acids 125-146.

25 *HLA restriction*

The HLA restriction of the TCC recognizing antigens encoded near 0.7 map units was determined in detail. Proliferation of TCC 4.2E1, specific for VP22 105-126, is inhibited

84% by anti-DP, but less than 20% by anti-DR or anti-DQ mAb. TCC 4.2E1 is from a DPB1*2001/DPB1*0402 heterozygous donor. Allogeneic EBV-LCL bearing DPB1*2001, but not DPB1*0402, present antigen (Table 3), establishing restriction by DPB1*2001. Proliferation of TCC 2.3, specific for UL50, was inhibited by anti-DR but not anti-DP or anti-DQ mAb. This clone is from a DRB1*0301/BRB1*0701 heterozygous donor. Allogeneic PBMC from a DRB1*0301 donor presented antigen, consistent with binding of antigenic peptide to this allele. However, presentation by the linked DR gene products DRw52 or DRw53, cannot be ruled out. Additional HLA restriction studies are summarized in Table 4.

10 Table 3. Determination of restricting HLA allele of lesion-derived CD4 TCC 4.2E1 and 2.3. Antigens were β -gal fusion proteins (Table 2) at 1:900 dilution. Data are delta cpm [^{3} H] thymidine incorporation compared to media, which was less than 500 cpm in each case.

<u>T-cell clone</u>	<u>antigen</u>	<u>APC</u>	<u>HLA type¹</u>	<u>delta cpm²</u>
4.2E1	1.1.3	autologous EBV-LCL	DPB1*0402, 2001	30,719
		AMAI EBV-LCL	DPB1*0402	2,732
		ARENT EBV-LCL	DPB1*2001	26,218
2.3	50.583.44	autologous PBMC	DRB1*0301, 0701	8,964
		allogeneic PBMC A	DRB1*0701, 1001	45
		allogeneic PBMC B	DRB1*0301, 1301	19,223

¹HLA type at the HLA class II locus as determined by inhibition with mAb.

15 ²In comparison to pUEX2 control protein (1:1000 dilution) with the same APC, which caused less than 500 cpm [^{3} H]thymidine incorporation in each case.

20 Table 4. Cytolytic activity of lesion-derived, tegument-specific CD4 TCC with summary of fine specificity and HLA restriction. Results are percent specific release at an effector to target ratio of 20:1 except ESL4.34 (10:1). Auto=autologous EBV-LCL as target cells;

allo=allogeneic EBV-LCL mismatched at the relevant HLA locus (if known) or mismatch at HLA DR and DQ.

TCC	specificity ¹	HLA restriction ²	cytolysis assay target					
			auto HSV-2	auto peptide	auto mock	allo HSV-2	allo peptide	allo mock
4.2E1	VP22 105-126	DPB1*200 1	20.7	44.2	-4.1	-2.9	-1.7	4.6
ESL4.9	VP22 187-206	DR ³	-0.6	20.2	1.3	0	0	0
1.L3D5.10.8	VP22 125-146	DR ⁴	1.1	61.1	-0.3	-0.4	-0.6	-0.4
1.L3D5.10.1 2	VP22 125-146	DR ⁴	2.5	57.6	1.6	-0.1	-2.5	-1.4

na=not available since epitope mapping was not done and synthetic antigenic peptide was not made.

5 nd=not done.

¹Indicates peptide used (1 µM) to load targets in CTL assay for selected TCC.

²Maximum extent of definition of HLA restricting locus and/or allele. Subjects RH and KM were typed serologically; others were typed at the DNA level.

10 ³Subject is heterozygous for HLA DRB1*0402 and DRB1*1301 and restricting allele has not been determined.

⁴Subject is heterozygous for HLA DRB1*0301 and DRB1*1102 and restricting allele has not been determined.

15 The HLA restriction of TCC BM.17 was studied in detail. Proliferation of TCC BM.17 and the similar clone SB.17 was inhibited 90% by anti-DQ, but less than 25% by anti-DR or anti-DP mAb. Donors BM and SB are heterozygous for HLA DQB1*0201/0501. At high concentrations of peptide, both DQB1*0201- and DQB1*0501 homozygous EBV-LCL appeared to present antigen to TCC BM.17.

CTL activity of tegument-specific CD4 T-cell clones

Cytotoxic activities of the CD4 TCC with newly and previously identified specificities were tested using EBV-LCL target cells (Table 4). All clones tested displayed cytolytic activity towards peptide-loaded target cells. Cytolytic activity against target cells infected
 5 with HSV-2 showed greater variability. VP22-specific TCC 4.2E1 was active, while VP22-specific TCC from other donors were not.

Discussion

HSV-specific T-cells selectively infiltrate recurrent genital HSV-2 lesions (D.M. Koelle et al., 1994, J. Infect. Dis., 169:956-961). Local CTL activity, with CD4 and CD8-mediated
 10 components, is correlated with viral clearance (D.M. Koelle et al., 1998, J. Clin. Invest. 101:1500-09). The antigens recognized by local HSV-specific T cells are diverse and in many cases unknown (D.M. Koelle et al., 1994, J. Virol., 68:2803-2810). This example documents recognition of tegument protein VP22.

The expression cloning system described herein works well with HSV. Genomic double
 15 stranded DNA can be used directly since introns are rare in the HSV genome. The same HSV-2 strain, HG52 (A. Dolan et al., 1998, J. Virol. 72:2010-2021) was used to screen candidate lesion-derived TCC and make protein libraries. The relatively low degree of strain variability (M.J. Novotny et al., 1996, Virology, 221:1-13) between HSV-2 strains in the donors and HG52 might rarely lead to omission of epitope(s) recognized
 20 *in vivo*; application to viruses with more strain variation would benefit from the use of autologous isolates.

Notably, reactivity with VP22 was detected in two independent expression cloning experiments with lesion-infiltrating TCC from two donors. VP22 reactivity was also detected during screening of the first available set of bulk lesion-infiltrating lymphocyte
 25 cultures. Ten additional clones from three patients have been negative with the disclosed fragments of UL49, UL21, and UL50.

Tegument antigens may be suitable targets for lesion-infiltrating CD4 T-cells because of their abundance. VP16 and VP22 are present in large amounts: on the order of 1.6×10^3 molecules of VP16 (Y. Zhang and J.L.C. McKnight, 1993, J. Virol., 67:1482-1492) and $2.5\text{-}2.8 \times 10^3$ molecules of VP22 (J. Leslie et al., 1996, Virology, 220:60-68) are
 5 incorporated into each virion in HSV-1.

Because polypeptides expressed as C-terminal fusion to VP22 can be co-transported into cells, expression of proteins as VP22 fusions may be of interest as a type of adjuvant preparation. This can be tested by expression of heterologous epitopes in VP22. VP16 and VP22 of HSV-1 are strongly, noncovalently associated in infected cells as shown by
 10 coimmunoprecipitation. These proteins co-localize in the perinuclear area of cells (G. Elliott et al., 1995, J. Virol., 69:7932-7941; G.D. Elliott et al., 1992, J. Gen. Virol., 73:723-736).

In summary, expression cloning has allowed discovery of novel HSV T-cell antigens. The in situ enrichment of antigen-specific CD4 T-cells in lesions allows study of the
 15 antigenic repertoire unbiased by secondary *in vitro* stimulation with antigen. The favorable characteristics of the HSV genome allow direct use of libraries of whole viral DNA. Tegument proteins are candidates together with membrane glycoproteins for use as HSV vaccines in humans.

20 Example 2: Efficacy of Full-length U_L49

This Example shows that the full-length U_L49 protein is effective at stimulating T cell proliferation. The data demonstrate the antigenicity of full-length U_L49 expressed in *E. coli* and in Cos-7 cells. These results confirm that the antigens described hereinabove were accurately identified.

25 To express full-length U_L 49 protein of HSV-2 in a prokaryotic system, the gene was cloned by PCR from DNA prepared from HSV type 2 strain HG52 using primers GGAAGATCTACCTCTCGCCGCTCCGTCA (SEQ ID NO: 5) at the 5' end of the

gene and CCGGAATTCTTGTCTGTCGTCTGAACGCG (SEQ ID NO: 6) at the 3' end of the gene. PCR product was digested with *Bgl* II and *Eco*R I and cloned into the *Bgl* II and *Eco*R I sites in the TA cloning vector pcR2.1-Topo (Invitrogen). The gene was then subcloned into the vector pTrcHisB (Invitrogen) and then into pGEX-2T (Pharmacia). The sequence of the HSV-2 UL49 clone had one coding mutation compared to the published sequence (Dolan 1998): amino acid 244 was mutated from serine to proline. The predicted amino acid sequence of the expressed protein also is missing the initial methionine. UL49 contains an N-terminal fusion domain derived from vector pGEX2T. This plasmid is named pGEX2T-UL49HSV2.

To make prokaryotically expressed full length UL49 of HSV-2, pGEX2TU-L49HSV2 or control empty vector was transformed into *E. coli* strain BL21. Bacteria in log-phase growth were adjusted to an OD₆₀₀ of 0.4 in LB-ampicillin media. To some tubes isopropyl beta-D-thiogalactopyranoside (IPTG) was added to 0.3 mM. Bacteria were cultured for 1.5 hours at 37°C with rotation. Bacteria were collected by centrifugation and washed 3X in PBS containing 1 mM EDTA, heated to 65°C for 10 minutes, and washed twice more with PBS, and resuspended at approximately 1 X 10⁹ bacteria/ml in T-cell medium. Heat-killed bacterial suspensions were used as test antigen.

To express full-length UL49 protein of HSV-2 in a eukaryotic system, the gene was separately re-amplified by polymerase chain reaction using a high-fidelity DNA polymerase with proof-reading function. The same primers and template were used. The gene was cloned directly into the *Bgl* II and *Eco*R I sites of pEGFP-C1 (Clontech). The entire UL49 gene was sequenced and agreed with published sequence. The predicted amino acid sequence of the expressed protein is identical to that predicted for viral UL49 except that the initial methionine at amino acid 1 is missing. A N-terminal fusion domain derived from vector pEGFP-C1 is also predicted to be expressed. This plasmid is named pEGFP-C1-UL49HSV2.

To make eukaryotically expressed full length UL49 of HSV-2, pEGFP-C1-UL49HSV2 plasmid DNA or pEGFP-C1vector control DNA was transfected into Cos-7 cells by

lipofection. After 48 hours, cells were scraped and sonicated and a supernatant and pellet phase prepared. Cells from a 9.4 cm² dish were used to prepare 300 microliters of supernatant. The pellet from a 9.4 cm² dish was resuspended in 300 microliters medium. Supernatant and pellet preparations were used as test antigens.

- 5 These test antigens were added to assay wells (96-well, U-bottom) in 200 microliters of T-cell medium containing 1×10^5 autologous irradiated peripheral blood mononuclear cells (PBMC) per well and 1×10^4 lesion-derived CD4-bearing T-cell clone ESL4.9 for UL49 (Koelle et al, 1994 and 1998). Assays were performed in duplicate or triplicate. After three days, ³H thymidine incorporation was measured as described in Example 1.
- 10 Results are expressed as stimulation index (mean cpm ³H thymidine incorporation test antigen/mean cpm ³H thymidine incorporation media control) and delta cpm (mean cpm ³H thymidine incorporation test antigen minus mean cpm ³H thymidine incorporation media control). Positive and negative control antigens were run as indicated and as described in Example 1.
- 15 Table 5. Antigenicity of full-length HSV-2 UL 49 expressed prokaryotically in *E. coli* BL21

<u>antigen</u>	<u>final dilution</u>	<u>delta cpm</u>	<u>stimulation index</u>
UV HSV-2	1:100	26,823	386
heat-killed pGEX2	1:4	-11	0.84
heat-killed pGEX2	1:40	-25	0.64
heat-killed pGEX2	1:400	-8	0.89
heat-killed pGEX2- UL49HSV2	1:4	9,413	135
heat-killed pGEX2-	1:40	10,526	152

UL49HSV2			
heat-killed pGEX2- UL49HSV2	1:400	5,021	73

Table 6. Antigenicity of full-length HSV-2 U_L 49 expressed eukaryotically in Cos-7 cells

<u>antigen</u>	<u>final dilution</u>	<u>delta CPM</u>	<u>stimulation index</u>
UV-mock virus	1:100	-4	0.96
UV HSV-2	1:100	46,510	470
supernatant of control-transfected cells	1:4	8	1.08
pellet of control-transfected cells	1:4	131	2.32
supernatant of U _L 49-transfected cells	1:4	1,512	16.3
pellet of U _L 49-transfected cells	1:4	84,951	859
pellet of U _L 49-transfected cells	1:40	35,753	362
pellet of U _L 49-transfected cells	1:400	29,854	302

These results show that HSV-2 protein U_L49 retains its immunogenicity when expressed
5 as a full-length protein. U_L49 was studied in both prokaryotic and eukaryotic systems.

Example 3: Prevalence of Antigens in Population

This example supports the utility of preventative and therapeutic uses of the antigens of the invention by demonstrating the prevalence of responses to these antigens among the population. To do this, seven individuals who were HSV-2 infected as documented by

type-specific serology were surveyed. These individuals were different from the individuals from whom the index T-cell clones were recovered from HSV-2 lesions.

For each subject, PBMC were isolated and plated at 2×10^6 cells/well in 2 mls of T-cell medium in 24-well plates and stimulated *in vitro* with a 1:500 dilution of UV-inactivated HSV-2 strain 333 for five days. At that time, 40 units/ml recombinant human IL-2 was added for an additional five to six days, giving rise to a short-term, HSV-specific cell line termed a B1 cell line.

Reactivity to individual HSV-2 proteins was assessed as follows. Proliferation assays were set up on 96-well round bottom microtiter plates, and each condition was performed in triplicate. To each well, 1×10^5 autologous irradiated (3300 rad gamma) PBMC were added as antigen presenting cells. To each well, 1×10^4 B1 cells were added. The following control substances were added: media, UV-treated mock virus preparation diluted 1:500, UV-treated HSV-2 strain 333 diluted 1:500, glycoproteins B or D or VP16 protein of HSV-2 (purified) at 4 micrograms per ml final concentration. The response to UV-treated HSV-2 was expected to be positive and served as a positive control for the viability and overall specificity of the cells. Glycoproteins B and D and VP16 were previously shown to be targets of HSV-specific T-cells (D. M. Koelle et al., 1994, J. Virol 68(5):2803-2810).

For the newly discovered antigen UL49, the cloning of the full-length gene and its expression in the eukaryotic Cos-7 system was as described above, as was the preparation of control antigens based on the empty vector. The supernatant and pellet after sonication of transfected Cos-7 cells was used at a final dilution of 1:20 in triplicate proliferation assays.

Positive responses were scored if the stimulation index (mean cpm ^3H thymidine incorporation for test antigen/mean cpm ^3H thymidine incorporation for relevant control antigen) was greater than or equal to 4.0. For UV HSV-2 antigen, the relevant control antigen was UV-mock virus. For gB2, gD2, and VP16, the control was media. For the new antigens expressed in Cos-7 cells, the control antigen was either the pellet or

supernatant of Cos-7 cells transfected with control empty vector. Results are shown in Table 7. Reactivity with each of the newly discovered antigens was documented in at least one study subject. Overall, reactivity with UL 49 was observed more frequently and similar to that for the known antigens gB2 and gD2. These data provide support that human individuals, in addition to the index subjects in whom the T-cell reactivity was originally described, are capable of reacting to these antigenic HSV-derived proteins.

Table 7. Antigenicity of known and of newly discovered HSV-2 antigens among a group of seven randomly chosen HSV-2 infected immunocompetent adults.

	<u>ANTIGEN</u>				
	<u>HSV-2</u>	<u>gB2</u>	<u>gD2</u>	<u>VP16 of HSV-2</u>	<u>UL 49 of HSV-2</u>
n	7	5	5	0	5
%	100	71	71	0	71

10 Example 4: Detection of HSV-specific CD8 CTL in recurrent genital HSV-2 lesions

This example demonstrates that specific CD8 CTL localize to genital HSV-2 lesions. This is shown by serial lesion biopsy studies of recurrent genital HSV-2 lesions using cells that have encountered antigen/APC *in situ* and are not restimulated with antigen *in vitro* prior to readout assays.

15 To study the cDNA species derived from the positive genomic clone containing portions of ICP0 (*Results*), COS-7 cells (100 mm²) were transfected with the ICP0 genomic clone, and total RNA was prepared after 48 h. The primer used for cDNA synthesis (TGCTCTAGAGACTCGATCCCTGCGCGTCGG; *Xba*I site underlined) (SEQ ID NO: 7) was from the 3'-end of the HSV-2 DNA in the ICP0 genomic clone. Moloney
20 murine leukemia virus reverse transcriptase (Life Technologies) was used per the

manufacturer. To examine splicing, PCR used *pfu* cDNA polymerase, the above 3'-primer, and 5'-primer TAAGGTACCTGAACCCCGGCCCGGCACGAGC (SEQ ID NO: 8) (*Kpn*I site). To isolate exon 1 (Dolan, A. et al., 1998, J. Virol. 72:2010) of ICP0, PCR used the same 5'-primer and 3'-primer

5 TGCTCTAGACCAGGCGTGCGGGGCGGCGGG (SEQ ID NO: 9) (*Xba*I site).

Reaction conditions were individually optimized. Product was digested with *Acc*65I and *Xba*I, gel purified, and ligated into similarly treated pCDNA 3.1-His-B, and in-frame insertion was confirmed by sequencing.

Full-length U_L 47 of HSV-2 was cloned by PCR into pCDNA3.1/His-C using 5'-primer
10 CTAGGATCCCCTCCGGCCACCATGTCC (SEQ ID NO: 10) and 3'-primer
CGATCTAGACCTATGGGCGTGGCGGGC (SEQ ID NO: 11) (*Bam*HI and *Xba*I sites underlined). Full-length U_L 46 of HSV-2 was cloned by PCR into pCDNA3.1/His-C with 5'-primer CGAGGATCCGTCTCCGCCATGCAACGCCG (SEQ ID NO: 12) and
15 3'-primer CGCTCTAGATTTTAATGGCTCTGGTGTCG (SEQ ID NO: 13) (*Bam*HI and *Xba*I sites underlined). Similarly, a construct expressing aa 1–590 of U_L 47 was made by PCR, using the above 5'-primer, an appropriate 3'-primer, and pCDNA3.1/His-C. Expression of aa 1–535 and 536–696 of U_L 47 was driven by constructs derived from full-length U_L 47 using a naturally occurring *Not*I site at aa 535. In-frame vector-HSV-2 fusion at the 5'-end of the HSV-2 DNA was confirmed by sequencing in each case.

20 Results

Table 8. CTL activity and HLA restriction of CD8 clones, and initial results of expression cloning¹

	CD8 CTL Clone		
	1874.1991.22	5101.1999.23	5491.2000.48
<i>Antologous targets</i>			
Mock	1.2	6	1.2
HSV-1	0.1	0	2.3
HSV-2	38.3	56.6	63.2
HSV-2 hr259 (ICP4 ⁻)	21.3	41.0	ND
HSV-2/actinomycin D	45.1	35.8	49.2
<i>HLA-mismatched targets</i>			
Mock	0	2.5	9.8

HSV-2	0	2.1	7.0
<i>HLA restriction testing</i>			
Matched allele	A*0201	A*0201	B*0702
Mock	3.3	0	5.1
HSV-2	65.2	33.4	69.1
<i>Specificity</i>			
Positive genomic clone	C:1:F1:C7	C:2:C10:B9	UL49-pEGFP-C1
Nucleotides	102,875–101,383	102,943–102,876	107,149–106,247
Predicted HSV-2 ORF(s)	UL 47 300–696 ... UL 46 1–71	UL 47 278–298	UL 49 1–300

¹ Data are percent specific release in ⁵¹Cr release assays at E:T 20:1. For actinomycin D experiments, target cells infected with wild-type HSV-2 were assayed in the presence of 5 µg/ml actinomycin D from 0.5 h before infection through the assay. To assess HLA restriction, allogeneic EBV-LCL were either mismatched at HLA-A and -B or matched with the index subject at only the indicated HLA class I allele. The positive HSV-2 genomic clones are listed by indicating the positive pCDNA3.1/His A, B, or C library: positive library plate: positive library well:positive final well. For 5491.2000.48, full-length UL 49 of HSV-2 in pEGFP-C1 (Clontech) was positive. The nucleotide numbers and predicted amino acid numbers within the antigenic HSV-2 DNA fragments are given as reported for the HSV-2 strain HG52 genomic sequence (28).

Recognition of tegument HSV-2 Ags by CD8 T cells

CD8 clone 5101.1999.23 recognized COS-7 cells co-transfected with HLA A*0201 and a HSV-2 *San3aI* fragment from bp 102,943–102,876 (Dolan, A. et al., 1998, J. Virol. 72:2010) (Table 8). The predicted fusion protein contains HSV-2 UL 47 aa 278–298. Reactivity with UL 47 was confirmed by cotransfection of A*0201 and full-length HSV-2 UL 47 (Table 9).

Table 9. Confirmation and localization of epitopes recognized by CD8⁺ clones¹

T Cell Clone	HSV-2 ORF and Predicted Amino Acids	HLA cDNA	IFN-γ (pg/ml)
5101.1999.23	None	None	0
	UL 47 aa 1–696 (full length)	None	0
	None	A*0201	0

	U _L 47 aa 1–696	A*0201	>3000
1874.1991.22	None	None	0
	U _L 47 aa 1–696	None	0
	None	A*0201	0
	U _L 46 aa 1–722 (full length)	A*0201	0
	U _L 47 aa 1–696	A*0201	2984
	U _L 47 aa 1–535	A*0201	0
	U _L 47 aa 1–590	A*0201	>3000
	U _L 47 aa 536–696	A*0201	>3000
1874.1997.51	Genomic, nucleotides 1858–3022	None	0
	None	B*4501	0
	Genomic, nucleotides 1858–3022	B*4501	>600
	ICP0 exon 1 cDNA aa 1–25	B*4501	3.2
	ICP0 exon 1/start of exon 2 cDNA aa 1–105	B*4501	>600

¹ The indicated CD8 CTL clones were reactive with HSV-2 genomic clones indicated in Table 8. COS-7 cells were transfected with HLA cDNA and HSV-2 DNA or cDNA as shown. T cell activation was detected by IFN- γ secretion, reported as the mean of duplicate wells. Two CTL clones are shown to react with U_L47. The epitope recognized by clone 1874.1991.22 is localized to aa 535–590 of U_L47, and the epitope recognized by clone 1874.1997.51 is localized to aa 26–105 of ICP0. Values are mean of duplicate IFN- γ secretion into the medium as measured by ELISA. ORF, Open reading frame.

The CD8 T cell clone 1874.1991.22 recognized COS-7 cells cotransfected with HLA A*0201 and a HSV-2 *Sau*3aI fragment from bp 102,875–101,383 (Table 8). This fragment was predicted to contain the DNA encoding U_L47 aa 300–696, intervening DNA, and then aa 1–71 of U_L46. Analysis of the 5'-vector-insert junction in C:2:C10:B9 revealed out-of-frame translation of the initial U_L47 DNA. The insert is expected to contain the U_L46 promoter. The epitope, therefore, could be encoded by U_L46. C:2:C10:B9 also contains potential sites of internal initiation of translation within U_L47. U_L46 and U_L47 were assayed separately in the COS-7 cotransfection assay (Table 9). U_L47 was active, whereas U_L46 was not. Truncation analysis localized the epitope to aa 535–590 (Table 9).

The specificity of CD8 clone 5491.2000.48 was determined with a panel of partial- and full-length HSV-2 genes. The HSV-2 genes studied were previously shown to be

recognized by CD4 T cell clones (See U.S. Patent 6,375,952, issued April 23, 2002). Only HSV-2 U_L49, when cotransfected with HLA B*0702, stimulated IFN- γ release by clone 5491.2000.48 (Fig. 5).

5 HSV-2 gene U_L47 encodes protein VP13/14, whereas U_L49 encodes VP22; both tegument proteins are loaded into the cytoplasm on virion binding and entry. The small genomic HSV-2 fragment of U_L47 recognized by clone 5101.1999.23 was scanned for peptides fitting the A*0201 binding motif (<http://134.2.96.221/> and http://birnas.dcrn.nih.gov/molbio/hla_bind/). Peptide U_L47 (HSV-2) 289–298 had a 50% effective concentration (EC₅₀) in the 1–10 nM range in cytotoxicity assays (Fig. 6).
 10 U_L47 535–590 (Table 9) was similarly analyzed. Peptide 551–559 was active at 1 nM (Fig. 6). Potential HLA B*0702-binding peptides in U_L49 of HSV-2 were synthesized, and two (aa 47–55 and 14–22) were active at 1 μ M. Titration (Fig. 6) showed that U_L49 49–55 was highly active, with an EC₅₀ of <10 nM, whereas U_L49 14–22 had activity only at 1 μ M. The antigenic peptides in U_L47 and U_L49 contain significant amino acid sequence
 15 differences from the corresponding predicted HSV-1 peptides (Dolan, A. et al., 1998, J. Virol. 72:2010; McGeoch, D. J. et al., 1988, J. Gen. Virol. 69:1531), explaining type-specific recognition of HSV-2 (Table 8).

Recognition of immediate early HSV-2 protein ICP0 by CD8 T cells

For clone 1874.1997.51, positive reactions to plasmid pools were present in each library.
 20 The active plasmids in each library contained a genomic *Sal*3AI fragment from nucleotides 1858–3022 (Dolan, A. et al., 1998, J. Virol. 72:2010). Nucleotide 2007 listed as T in the published sequence was read as C. In addition to 445 bp of 5'-untranslated sequence, all of predicted exon 1, intron 1, and the first 234 bp of predicted exon 2 of ICP0 were present, preliminarily identifying the antigen as ICP0. Because alternative
 25 splicing of HSV-1 ICP0 has been documented at both the RNA and protein levels (Weber, P. C. et al., 1999, Virology 253:288; Carter, K. L., B. Roizman, 1996, Proc. Natl. Acad. Sci. USA 93:12535), the Ag-encoding mRNA species was first identified in COS-7 cells to determine how the ICP0 genomic clone was spliced in this system. COS-7 cells

were transfected with genomic clone C:1:H3:B8 (Table 8), and cDNA was synthesized from total cellular RNA followed by PCR designed to amplify the spliced transcript. The size of the PCR product (~300 bp) was consistent with the splicing out of intron 1. Sequencing showed a slight difference from the reported splice point for mature HSV-2 ICP0 mRNA. Three base pairs encoding aa Q26 were missing. Q26 was retained for peptide numbering (below). To determine whether the antigenic peptide lay within exon 1 or exon 2, PCR was repeated with specific primers. The exon 1-partial exon 2 cDNA, but not exon 1 cDNA, was stimulatory for T cell clone 1874.1997.51 (Table 9), localizing the epitope to aa 26–105 in exon 2. Reactivity was confirmed in CTL assays using a recombinant vaccinia virus expressing ICP0. At E:T 20:1, lysis of vaccinia ICP0-infected target cells was 52.1% compared with 2.3% for vaccinia wild type.

Two reported HLA B45-restricted epitopes, AEEAAGIGIL (SEQ ID NO: 14) and GAETFYVDGA (SEQ ID NO: 15), share with the B44 supertype a preference for negatively charged and hydrophobic amino acid side chains at the P2 and P9 anchor positions. ICP0 (HSV-2) 92–105, containing this motif, was active at 1 μ M. Truncation yielded ICP0 (HSV-2) 92–101, with an EC₅₀ in the 1 nM range (Fig. 6).

Recognition of skin-derived fibroblasts and keratinocytes by CD8 CTL clones

Within lesions, HSV-2 is mainly present in keratinocytes. How MOI (amount of virus), time of infection, and pretreatment with IFN- γ influenced lysis of dermal fibroblasts and keratinocytes was investigated. For fibroblasts (Fig. 7), in the absence of IFN- γ pretreatment, infection for 2 h led to detectable lysis, which increased with increasing MOI. Lysis was undetectable (<5% specific release at E:T of 20:1) after overnight infection with MOI 1, 5, or 25. With IFN- γ pretreatment, lysis was generally increased, but 2-h infection was still superior. HLA-mismatched target cells were not lysed, even after peptide loading.

Keratinocytes showed some similarities and differences from fibroblast as target cells (Fig. 7). IFN- γ pretreatment generally increased recognition, without leading to lysis of control cells. In contrast to fibroblasts, 18-h infection was generally required. Weak

cytolysis of cells infected for 2 h was noted only for IFN- γ -pretreated targets. Chromium release again correlated directly with the amount of infectious virus added, because no specific lysis was noted at MOI 1 or 5.

TAP dependence of Ag processing for recognition by HSV-2 tegument protein epitopes by CD8 CTL

- 5 For each of the three CD8 clones studied, lysis of TAP-deficient cells after HSV-2 infection was greatly reduced in comparison to wild-type EBV-LCL (Table 10). Greater than 90% of each of the TAP-deficient cell lines, as well as control wild-type LCL, were permissive for viral infection and protein synthesis as evaluated by flow cytometry using mAb specific for envelope glycoprotein gD. Peptide loading was able to sensitize the
- 10 TAP-deficient cells, confirming HLA class I expression.

Table 10. TAP dependence of processing of HSV-2 tegument for presentation to CD8 T cells¹

Target Cells	Mock	Peptide	HSV-2
CD8 clone 1874.1991.22			
<i>Controls</i>			
1874	2.5	54.8	30.7
5491	2.0	-2.5	-1.2
<i>TAP (-)</i>			
721.174	-3.9	90.0	1.3
T2	0.7	94.7	2.2
CD8 clone 5101.1999.23			
<i>Controls</i>			
1874	0.8	52.5	18.2
5491	1.7	-2.1	-1.2
<i>TAP (-)</i>			
721.174	0	31.7	2.9
T2	-0.7	71.0	0.8
CD8 clone 5491.2000.48			
<i>Controls</i>			
1874	0.8	-2.7	3.6
5491	0.2	68.3	21.2
<i>TAP (-)</i>			
T2/B7.63	-0.4	57.8	3.4
T2	0.2	0.4	3.6

¹ Data are percent specific release in ⁵¹Cr release assays at E:T 20:1. The first two clones are HLA A*0201-restricted. Lysis of the A*0201-bearing wild-type EBV-LCL 1874, but not non-A*0201 EBV-LCL 5491, was detected after peptide loading (U_L47 551–559 for clone 1874.1991.22; U_L47 551–559 for clone 5101.1999.23; 1 μM, 90 min) or HSV-2 infection (MOI 10, 18 h). In contrast, peptide loading, but not HSV-2-infection, was able to sensitize TAP-deficient cell lines. Similar data are shown for the third clone, a HLA B*0702-restricted, U_L49-specific CTL clone and peptide U_L49 49–57, using the B*0702 autologous EBV-LCL, 5491, the non-B*0702 EBV-LCL, 1874, and the TAP-deficient, HLA B*0702-containing transfectant T2/B7.63. As an additional control, T2 cells, which do not express B*0702, were not lysed after peptide loading.

Discussion

HSV-2 causes considerable morbidity and mortality, especially in neonates. Because of the chronic nature of the infection, the limitations of antiviral therapy, and the frequency with which transmission is caused by asymptomatic shedding of the virus, vaccination is likely to be required to reduce new HSV-2 infections. The recent report that vaccination with a specific adjuvant and an envelope glycoprotein induced partial protection in HSV-1/HSV-2-seronegative women highlights both the potential efficacy of vaccination and the need for improved formulations and markers of effective immunity.

Little is known about the specificity of human HSV-2-specific CD8 CTL. The two published epitopes are type-common peptides within glycoproteins B and D. At the nonclonal level, experiments using restimulation of PBMC, drug blocks, and vaccinia recombinants show that HSV-1 ICP4, ICP27, ICP0, all immediate early proteins, HSV-1 early protein ICP6, and possibly other true early proteins may be targets of human CTL. HSV-1 early protein thymidine kinase (tk) is recognized by CD8 clones from PBMC of subjects treated with *tk*-transfected autologous cells, but this is likely a primary immune response. A PBMC-derived CD8 T cell clone specific for a melanoma-associated protein (Melan A/MART-1) also reacted with a peptide from HSV-1 glycoprotein C.

U_L49 encodes VP22, a tegument protein required for viral replication. U_L49 protein is also abundant in virions and delivered into the cytoplasm by virus entry. Lysis of EBV-LCL by tegument-specific CD8 CTL was not inhibited by blockade of gene transcription or infection with a replication-incompetent virus, consistent with the processing and
 5 presentation of preformed virion input protein.

TAP-independent processing has been reported in other viral systems. The examination of three discrete epitopes in tegument proteins did not reveal evidence for TAP-independent Ag processing of HSV epitopes. The CD8 response seems to "evade the evasion," at least in the cases examined to date, while continuing to rely on TAP for Ag
 10 processing.

Most studies of clonal CD8 responses have used EBV-LCL as target cells. These cells are relatively resistant to HSV-mediated class I down-regulation. For dermal fibroblasts, it was found that a short time of infection (2 h) was adequate for target cell sensitization for lysis by tegument protein-specific CTL. Because the U_L47 and U_L49 tegument proteins
 15 are synthesized with "late" kinetics, typically starting after 6 h or more of viral infection, these data are also consistent with recognition of preformed Ag in fibroblasts. Lysis was MOI dependent. Because HSV preparations typically contain a large number of defective particles, it is likely that tegument proteins were also being delivered into fibroblasts by noninfectious particles. After 18 h of infection, the fibroblasts were not lysed, regardless
 20 of MOI, similar to previous results with CD8 CTL clones of unknown fine specificity. IFN- γ pretreatment was able to partially restore lysis of 18-h-infected cells. In contrast to fibroblasts, recognition of keratinocytes after 18 h of infection was superior to recognition after 2 h of infection. The reason for the difference between fibroblasts and keratinocytes is unknown. IFN- γ pretreatment was able to restore some lysis of 2-h-
 25 infected cells, and further improved recognition of 18-h-infected cells.

Tegument proteins have not previously been described as targets of the HSV-specific CD8 T cell response. CD4 responses to HSV-1 U_L47 have been detected in HSV-mediated acute retinal necrosis. CD4 responses to U_L49 are commonly detected among

lesion-infiltrating HSV-2-specific clones. Because responses to UL49 are also present in the cornea in herpes stromal keratitis in humans, a disease that may be driven by pathogenic Th1-like T cells, caution is warranted in using this protein as a vaccine. Overall, UL49 is the only known HSV-2 protein recognized by both CD4 and CD8 T cell clones recovered from herpetic lesions. A unique intercellular transport pathway allows highly efficient uptake of soluble UL49 protein into a variety of epithelial cell types which could also intersect antigen processing pathways.

In summary, reactivity of lesion-infiltrating, HSV-2 type-specific CD8 T cell clones with the tegument proteins encoded by genes UL47 and UL49 (VP13/14 and VP22, respectively), and ICP0, are described for the first time. The data are consistent with a modulatory effect of ICP47 and/or *gB*s on the CD8 response to HSV. TAP function, but not viral gene transcription, is required for recognition by UL47- and UL49-specific clones, consistent with processing of preformed virion input protein. Tegument-specific CD8 clones were able to recognize skin-derived fibroblasts and keratinocytes. Responses were also detectable in the PBMC of additional subjects.

Example 5: HSV-2 15-mer Peptide Screening with CD8+ T Cells

HSV-2 seropositive donors were obtained (AD104, AD116, AD120, D477, HV5101, JH6376, EB5491, TM10062). Donors HV5101 and EB5491 experience frequent anogenital lesion recurrences. Donors JH6376 and TM10062 experience few or no anogenital lesion recurrences. Leukopheresis PBMC were obtained from each donor after informed consent. Donor PBMC were HLA-typed by low resolution DNA typing methodology. Synthetic peptides (15 amino-acids in length and overlapping in sequence by 11 amino-acids) were synthesized that spanned the following HSV-2 polypeptides: UL47 (aa 1-696), UL49 (aa 1-300), ICP27 (aa 1-512). Peptides (5 mg each) were delivered in lyophilized form in glass vials and dissolved at a concentration of 10 mg/ml in DMSO, transferred to sterile cryovials and stored at -20 degrees C. The peptides were screened with CD8+ T cells purified from adherent

macrophage-depleted PBMC. CD8+ T cells were purified by depletion of non-CD8+ cells using a commercial MACS bead kit (Miltenyi). CD8+ T cells purified in this manner were generally > 95% CD8+CD3+CD4-, as measured by flow cytometry (FACS). Peptides were screened by 24-hour co-culture of CD8+ T cells (2 x 10e5/well) and autologous dendritic cells (1 x 10e4/well) and peptides (10 µg/ml each) in 96-well ELISPOT plates that had been pre-coated with anti-human IFN-γ antibody 1D1K (mAbTech). Peptides were initially screened as pools of ≥ 10 peptides. ELISPOT plates were subsequently developed per a standard protocol. The number of spots per well was counted using an automated video-microscopy ELISPOT reader. Peptides in pools scoring positive were subsequently tested individually in a second ELISPOT assay. For AD116, the novel peptides U_L49/21-35 (#6) and U_L49/193-208 (#49) scored positive both pooled and individually. AD116 also recognized the previously described B*0702-restricted epitope U_L49/49-57 contained in 15-mer peptides U_L49/45-59 (#12) and U_L49/49-63 (#13). D477, HV5101, and JH6376 T cells recognized the previously described HLA-A*0201-restricted epitopes U_L47/289-297 and U_L47/550-559 contained in 15-mers #73/#74 and #137/#138, respectively. EB5491 T cells recognized the previously described B*0702-restricted epitope U_L49/49-57 epitope contained in 15-mer peptides U_L49/45-59 (#12) and U_L49/49-63 (#13). D477 scored positive for peptide pool U_L49 (#11-20). TM10062 did not score positive on any peptide pool from U_L47 or U_L49.

Donor HLA Types

Donor:	<u>HLA-A</u>	<u>HLA-B</u>	<u>HLA-C</u>
AD104	24, 33	46, 58	01, 0302
AD116	0206, 24	0702, 35	0702, 1203
AD120	0211, 3303	1505, 4403	0303, 0706
D477	0201, 2501	1501, 5101	0304, 12
HV5101	0101, 0201	0801, 57	06, 0701
JH6376	0201, 03	07, 44	05, 07

EB5491	01, 26	07, 08	07
TM10062	0201, 26	14, 27	01, 08

CD8+ T cell peptide-screening hits

	AD104 US3 #33	HSV-2 AIDYVHCKGIIHRDI (SEQ ID NO: 16)
5		HSV-1 AVDYIHRQGGIIHRDI (SEQ ID NO: 17)
	AD116 UL47 #86	HSV-2 AVPLLSAGGAAPPHP (SEQ ID NO: 18)
		HSV-1 AVPLLSAGGLVSPQS (SEQ ID NO: 19)
	UL49 #6	HSV-2 ELYYGPVSP-ADPESP (SEQ ID NO: 20)
		HSV-1 DLYYTPSSGMASPDSP (SEQ ID NO: 21)
10	UL49 #12	HSV-2 PMRARPRGEVRFLHY (SEQ ID NO: 22)
		HSV-1 QRSARQRGEVRFVQY (SEQ ID NO: 23)
	UL49 #13	HSV-2 RPRGEVRFLHYDEAG (SEQ ID NO: 24)
		HSV-1 RQRGEVRFVQYPESD (SEQ ID NO: 25)
	UL49 #49	HSV-2 VAGFNKRVFCAAVGR (SEQ ID NO: 26)
15		HSV-1 VAGFNKRVFCAAVGR (SEQ ID NO: 27)
	HV5101 UL47 #143	HSV-2 STAPEVGTYTPLRYAC (SEQ ID NO: 28)
		HSV-1 FTAPEVGTYTPLRYAC (SEQ ID NO: 29)

Figure 8 shows the results for AD116. Figure 9 shows the results for EB5491, TM10062 and HV5101. The peptide hit indicated with a “1” represents U_L49, amino acids 49-57, B*0702-restricted. Figure 10 shows results for AD104, AD116, AD120 and D477. The peptide hit indicated with a “1” represents peptide #54. The peptide hit indicated with a “2” represents peptide #49. The peptide hit indicated with a “3” represents U_L49, amino acids 49-57, B*0702-restricted. The peptide hit indicated with a “4” represents peptide #6.

Example 6: Detection of HSV-specific T-cell responses in cervical lymphocytes

Mucosal immune responses are segregated from PBMC, and localization of HSV-specific CTL to the mucosa of mice is associated with protection from vaginal inoculation. This example demonstrates that HSV-specific T cells, including CD8+
 5 cells, can be detected in cervical lymphocytes.

Cells from a representative cervical cytobrush specimen were collected during an active genital HSV-2 outbreak and expanded in bulk with PHA/IL-2, and subsequently analyzed for HSV-specific proliferative and cytotoxic responses. Proliferation and cytotoxicity assays used autologous PBMC or LCL as APC as
 10 described above for skin-derived lymphocytes. Anti-HLA class I mAb W6/32 or anti-HLA DR mAb L243 were used as described (Koelle DM et al., J. Virol. 1994, 68:2803-10; Koelle DM et al., J. Infect. Dis. 1994, 169:956-61). Antigen-specific proliferative responses and cytotoxic responses were present. Fractionation and mAb inhibition studies show a contribution of CD8 CTL to the cytotoxic response.

15 Example 7: Detection of HSV-specific T-cell responses in primary genital HSV-2 lesions

In this example, biopsy specimens were collected from a patient presenting with symptoms consistent with primary genital HSV-2 infection. The phenotypes of the collected cells were determined, and LIL and PBMC from the specimens were
 20 subjected to proliferative and cytotoxicity assays. The results show that the HSV-specific proliferative and cytotoxic responses of CTL present in primary genital HSV-2 lesions are typical of those detected during recurrent disease.

CW7477 developed dysuria, fever, buttock, and lower abdomen lesions three days after his last sexual contact. Lesions lasted 13 days and grew HSV-2. Acyclovir
 25 treatment was begun on day four of symptoms. Biopsies were done on days four and seven. Serostatus was atypical positive (only a few bands present on immunoblot) at day four, with more bands, but still less than most convalescent sera,

on day 26, by enhanced chemiluminescence (ECL; Dalesio J. and Ashley R., J. Clin. Microbiol. 1992, 30(4):1005-7) variant of type-specific HSV-2 immunoblot. The clinical and laboratory data were consistent with primary genital HSV-2 infection. Biopsy specimens were obtained on day four and seven of symptoms and bulk LIL
5 expanded with PHA/IL-2 as described above.

The phenotype of the expanded cells was split between CD4 and CD8 cells, with 15-25% CD3+/CD16,56+ cells and 5-10% TCR $\gamma\delta$ + cells in the LIL. In comparison, cells from normal skin had almost no CD16,56 (+) events and no TCR $\gamma\delta$ cells. The nature of the CD3+/CD16,56+ cells is unknown but these are frequently seen in
10 expanded LIL. The antibody cocktail has a combination of α CD16-PE and α CD56-PE.

The HSV-specific proliferative and cytotoxic responses were fairly typical of those detected during recurrent disease (Koelle DM et al., J. Clin. Invest. 1998; 101:1500-1508). Cross-reactive responses to HSV-1 and HSV-2 were present, as were antigen-
15 specific responses to HSV glycoproteins. Normal skin responses were low, and PBMC responses were developing by day 15.

Example 8: Identification of an ICP0 antigen recognized by HSV-specific CD8 CTL

This example demonstrates, via expression cloning, the antigenicity of ICP0. In particular, an epitope within amino acids 92-101 of ICP0 is identified. In addition,
20 the antigenicity of ICP0 is confirmed using vaccinia. The amino acid numbering uses the nomenclature and numbering of Dolan et al., J. Virol 1998, 72:2010-21. The methods used herein are described in U.S. Patent No. 6,413,518, issued July 2, 2002.

Results

25 All HSV-specific CD8 clones released IFN- γ in a specific manner. In addition, the utility of the interferon-gamma assay was examined as a confirmatory test for HLA

restriction. Clone RW51 specifically released interferon-gamma after exposure to Cos-7 cells transfected with HLA B*4501, but not with A*0201, and infection with HSV-2.

Table 12. Secretion of interferon-gamma of CD8 TCC RW51 in response to Cos-7 cells transfected with various DNAs (or peptide loaded at 1 μ M) measured by ELISA in pg/ml. Responses of 5×10^4 TCC to 7×10^3 Cos-7 cells checked at 24 hours.

<u>HLA class I</u> <u>cDNA</u>	<u>HSV-2 DNA or peptide</u>					
	<u>empty</u> <u>vector</u>	<u>pool</u> <u>A1:H3</u>	<u>clone</u> <u>A1:H3:B8</u>	<u>ICP0</u> <u>exon 1</u>	<u>ICP0</u> <u>exon 1,2</u>	<u>ICP0</u> <u>92-105</u>
empty vector	not done	not done	<2	<2	<2	<2
B*4501	<2	420	>600	<2	>600	1,100

To choose peptides efficiently, a HLA B45 binding motif was derived from B45-restricted peptides, and pool sequence from peptides eluted from B*4501. The motif is glutamic acid at position 2, hydrophobic at position 10 (P1 and P9 in "binding" nomenclature (Rammansee H-G, Current Opinion in Immunology 1995, 7:85-96)). Peptide ICP0 92-105 (AERQGSPTPADAQG; SEQ ID NO: 30) was active in CTL (Figure 14) and interferon-gamma (Table 12) assays. Other candidate exon 2 peptides were not. The high EC₅₀ value ($\sim 1 \mu$ M) may be due to the carboxy-terminus tail predicted to lie outside the peptide-binding groove and reduce binding to HLA B*4501. Vaccinia-ICP0 from B. Rouse (Manickan E et al., J. Virol. 1995, 69:4711-16) was grown and titered (Koelle DM et al., J. Virol. 1994, 68:2803-10). Clone RW51 specifically lysed vac-ICP0 targets (Figure 12). The availability of the vaccinia was fortuitous, and not required to confirm the result of expression cloning.

To narrow down the epitope, a peptide comprising amino acids 92-101 of ICP0 (AERQGSPTTP; SEQ ID NO: 31) was synthesized. The IC₅₀ for this peptide is between 1 and 10 nanomolar (Figure 13).

To confirm that patients with HSV-2 infection have T-cells reactive with the newly
 5 discovered T-cell antigen circulating in their peripheral blood, peripheral blood mononuclear cells (PBMC) from the patient from whom the lesion-derived clone RW51 was recovered were peptide stimulated. PBMC were cultured for three days at 2×10^6 cells per 1.88 cm² well in 2 ml of T-cell medium containing 1.0 µg/ml peptide HSV-2 ICP0 92-101. On the fourth day, IL-2 (32 units/ml) was added. On
 10 the eighth day, the cells were washed and restimulated in the same size well with an additional 2×10^6 autologous, irradiated (3300 rad gamma irradiation) PBMC, 1.0 µg/ml of the same peptide, and IL-2 (32 U/ml).

Responders were cultured for an additional nine days in the presence of IL-2 and expanded as necessary. Cytotoxicity assay was performed using autologous or HLA
 15 class I-mismatched LCL treated either with nothing, peptide HSV-2 ICP0 92-101 at 1 µg/ml for 18 hours, or infection with HSV-2 strain 333 at MOI 10 for 18 hours. The cytotoxicity assay was a standard four-hour ⁵¹Cr release assay.

The results (Figure 14) show that stimulation of PBMC with peptide HSV-2 ICP0 92-101 was able to stimulate cells with cytotoxicity towards HSV-2 infected cells,
 20 and that this activity was not present against HLA class I-mismatched cells. For comparison, the index T-cell clone RW51 was also used as an effector cell in this assay and displayed comparable, although slightly higher, cytotoxicity at the effector to target ratio of 10:1 shown in Figure 14.

25 Example 9: Identification of additional ICP0 antigens recognized by HSV-specific CTL

This example demonstrates, via use of a different population of CD8⁺ T cells from a different human subject, the specific recognition of amino acids 743-751 by lesion-

derived T cells. The recognition event involves HLA allele B*0702, which is relatively common (approximately 10%) in the human population. In addition, amino acids 288-307 of ICP0 have been found to be specifically recognized by lesion-derived T cells.

5 Example 10: ICP0 stimulation of CTL responses in additional HLA-B45 subjects

This example demonstrates that other HLA-B45 positive donors have detectable CD8+ T cell responses to the previously defined ICP0 92-101 peptide.

Peptide restimulation in bulk format are appropriate for sensitive detection of CTL, while lesion derived antigen (LDA) formats yield CTL levels, but require prolonged
 10 cell replication for detection. In this example, 4×10^6 PBMC in 2 ml T-cell medium were stimulated with 1 μ g/ml HSV-2 peptides, and IL-2 (10-30 U/ml) was added on day 3. On day 8, responders were washed and restimulated in 2 ml with 2×10^6 autologous irradiated PBMC, fresh peptide, and IL-2, and split as necessary until
 15 assay on day 14-16. For two HLA B*4501-bearing persons including the index subject, convincing HLA class-restricted CD8 CTL were detected that not only lysed peptide-loaded targets, but also killed HSV-2-infected targets and were inhibited by anti-class I mAb (Table 13).

Table 13. Lysis of HLA B*4501 LCL by PBMC stimulated with peptide HSV-2 ICP0 92-101, or (+) control clone RW.1997.51. Results are percent specific release
 20 in four-hour CTL assays at effector to target ratio of 10:1-20:1.

<u>effector</u>	<u>target</u> ¹						
	<u>RW</u> <u>mock</u>	<u>RW</u> <u>peptide</u> ¹	<u>RW</u> <u>HSV-2</u>	<u>RW HSV-</u> <u>2/ anti-</u> <u>class I</u> ²	<u>HV</u> <u>mock</u>	<u>HV</u> <u>peptide</u>	<u>HV</u> <u>HSV-2</u>
RW PBMC	1	45.3	48.2	12.2	0	-1	0
PO PBMC	0	54.9	33.5	5.8	4	-1	0
clone RW.1997.51	0	65.3	67.3	5.2	1	0	2

¹ Target LCL (RW=B*4501, HV=not B*4501) loaded with 1 µg/ml ICP0 92-101 for 90 minutes, or HSV-2 infection, MOI 5, 18 hours.

² Anti-HLA class I mAb W6/32 included at 10 µg/ml.

5 Example 11: Definition of additional T-cell epitopes in tegument protein UL48 (VP16)

Three epitopes within VP16, all HSV-2 type-specific were previously identified (K.R. Jerome et al., 1998, J. Virol., 72:436-441), and proliferative responses to full-length VP16 in bulk cultures of genital HSV-2 lesion-infiltrating lymphocytes from four of seven
 10 (57%) patients were detected (D.M. Koelle et al., 1998, J. Clin. Invest., 101:1500-09). Additional peptide epitopes were sought within VP16 by two strategies. The first strategy involved screening panels of clones recovered from lesion vesicle fluid for reactivity with recombinant VP16 of HSV-2 followed by epitope mapping with peptides. Peptides containing amino acids 185-197 and the overlapping pair 209-221 and 213-225
 15 were stimulatory for TCC RH.13 and KM.7, respectively. All other VP16 peptides tested were negative (<500 cpm). The second strategy involved using PBMC as starting material and secondary *in vitro* stimulation with recombinant baculovirus-derived VP16. Clones (BM.17 and SB.17) from two individuals recognized the same peptide (amino acids 437-449) as well as β-gal-VP16 fusion protein and whole virus. All three newly
 20 defined VP16 epitopes were type-common, shared by HSV-1 and HSV-2 whole virus preparations, as expected from sequence data (A. Cress and S.J. Triezenberg, 1991, Gene, 103:235-238).

Table 14. Epitopes within UL48 (VP16) of HSV-2 recognized by lesion- and PBMC-derived CD4⁺ TCC. Data are delta cpm [³H] thymidine incorporation compared to media, which was less than 500 cpm in each case.

<u>TCC</u>		<u>whole virus</u> <u>antigen</u>		<u>recombinant HSV-2</u> <u>protein¹</u>		<u>HSV-2 VP16 peptide</u>	
<u>name</u>	<u>origin</u>	<u>HSV-1</u>	<u>HSV-2</u>	<u>VP16 1-492</u>	<u>β-gal-VP16</u> <u>180-492</u>	<u>amino</u> <u>acids</u>	<u>delta cpm</u>
<i>newly reported epitopes</i>							
RH.13	lesion	3,340	3,407	32,991	nd	185-197	55,614
KM.7	lesion	6,093	5,847	5,627	nd	209-221	10,075
BM.17	PBMC	30,784	13,777	nd	45,958	437-449	79,723
SB.17	PBMC	2,207	4,187	nd	12,178	437-449	36,442
<i>previously reported epitopes</i>							
ESL4.34	lesion	256	8,780	17,302	nd	389-401	12,968
						393-405	95,942
ESL3.334	lesion	253	14,232	22,754	16,434	430-444	27,283
1A.B.25	lesion	414	33,493	24,919	41,123	431-440	38,664

¹VP16 1-492 (baculovirus-derived) was used at 1 µg/ml. β-gal-VP16 180-492 was used at 1:1,000 dilution.

²Peptides were used at 1 µM.

na=not available

nd=not done

10 Table 15. Cytolytic activity of lesion-derived, tegument-specific CD4⁺ TCC with summary of fine specificity and HLA restriction. Results are percent specific release at an effector to target ratio of 20:1 except ESL4.34 (10:1). Auto=autologous EBV-LCL as target cells; allo=allogeneic EBV-LCL mismatched at the relevant HLA locus (if known) or mismatch at HLA DR and DQ.

TCC	cytolysis assay target							
	<u>specificity</u> ¹	<u>HLA</u> <u>restriction</u> ²	<u>auto</u> <u>HSV-2</u>	<u>auto</u> <u>peptide</u>	<u>auto</u> <u>mock</u>	<u>allo</u> <u>HSV-2</u>	<u>allo</u> <u>peptide</u>	<u>allo</u> <u>mock</u>
<i>newly reported</i> epitopes RH.13	VP16 185- 197	DR ⁴	62.5	55.2	-0.9	9.6	0.3	1.8
KM.7	VP16 209- 221	DR ⁴	38.7	43.6	2.7	-2.2	4.3	-1.1
BM.17	VP16 437- 449	DQB1*050 1	10.1	28.5	-0.3	nd	nd	nd
SB.17	VP16 437- 449	DQB1*050 1	48.7	60.6	5.4	nd	nd	nd
<i>previously</i> <i>described</i> epitopes ESL4.34	VP16 393- 405	DRB1*0402	2.1	10.4	1.0	0.5	0.6	0.3
ESL3.334	VP16 430- 444	DQB1*030 2	12.3	33.6	0.7	1.4	0.3	2.2
1A.B.25	VP16 431- 440	DQB1*020 1	24.3	42.2	1.9	1.7	2.1	-0.4

na=not available since epitope mapping was not done and synthetic antigenic peptide was not made.

nd=not done.

¹Indicates peptide used (1 μ M) to load targets in CTL assay for selected TCC.

5 ²Maximum extent of definition of HLA restricting locus and/or allele. Subjects RH and KM were typed serologically; others were typed at the DNA level.

10 The HLA restriction of TCC BM.17 was studied in detail. Proliferation of TCC BM.17 and the similar clone SB.17 was inhibited 90% by anti-DQ, but less than 25% by anti-DR or anti-DP mAb. Donors BM and SB are heterozygous for HLA DQB1*0201/0501. At high concentrations of peptide, both DQB1*0201- and DQB1*0501 homozygous EBV-LCL appeared to present antigen to TCC BM.17. However, DQB1*0501 homozygous cells presented peptide much more efficiently than DQB1*0201

homozygous cells (Figure 19). Thus, three different but overlapping epitopes in VP16 431-449 are presented by HLA DQB1*0302, DQB1*0201, and DQB1*0501.

CTL activity of tegument-specific CD4 T-cell clones

Cytotoxic activities of the CD4 TCC with newly and previously identified specificities
 5 were tested using EBV-LCL target cells. All clones tested displayed cytolytic activity towards peptide-loaded target cells. Cytolytic activity against target cells infected with HSV-2 showed some variability. Among the seven VP16-specific T-cell clones tested, six displayed greater than 10% cytotoxicity towards HSV-2-infected target cells.

An additional epitope, included in amino acids 288-307 of UL48 (VP16)
 10 (RLRELNHIREHLNLPLVRSA; SEQ ID NO: 32), was demonstrated to have reactivity with a CD4+ T cell clone. This epitope is recognized in association with the HLA class II molecule DRBI*1501, which is fairly prevalent in most human populations.

15 Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended
 20 claims.

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

What is claimed is:

1. A pharmaceutical composition comprising a herpes simplex virus (HSV) polypeptide, wherein the polypeptide comprises a UL48, UL49 or ICP0 protein, and a pharmaceutically acceptable carrier.
- 5 2. The pharmaceutical composition of claim 1, wherein the protein comprises amino acids 185-197, 209-221, 288-307 or 430-449 of UL48 (SEQ ID NO: 2), amino acids 14-22, 21-35, 45-59, 49-57, 49-63, 105-190, 177-220 or 193-208 of UL49 (SEQ ID NO: 3), or amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 (SEQ ID NO: 1).
- 10 3. The pharmaceutical composition of claim 1, wherein the polypeptide is a fusion protein.
4. The pharmaceutical composition of claim 3, wherein the fusion protein is soluble.
5. The pharmaceutical composition of claim 1, further comprising an adjuvant.
- 15 6. A polynucleotide that encodes a polypeptide comprising an amino acid sequence consisting essentially of amino acids 185-197, 209-221, 288-307 or 430-449 of UL48 (SEQ ID NO: 2), amino acids 14-22, 21-35, 45-59, 49-57, 49-63, 105-190, 177-220 or 193-208 of UL49 (SEQ ID NO: 3), or amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 (SEQ ID NO: 1).
7. A vector comprising the polynucleotide of claim 6.
- 20 8. A host cell transformed with the vector of claim 7.
9. A method of producing an HSV polypeptide comprising culturing the host cell of claim 8 and recovering the polypeptide so produced.
10. An HSV polypeptide produced by the method of claim 9.

11. A pharmaceutical composition comprising the polynucleotide of claim 6 and a pharmaceutically acceptable carrier.
12. The pharmaceutical composition of claim 11, further comprising an adjuvant.
13. A pharmaceutical composition comprising a polynucleotide that encodes a UL48,
5 UL49 or ICP0 protein, and a pharmaceutically acceptable carrier.
14. The pharmaceutical composition of claim 13, further comprising an adjuvant.
15. A recombinant virus genetically modified to express an amino acid sequence consisting essentially of amino acids 185-197, 209-221, 288-307 or 430-449 of UL48 (SEQ ID NO: 2), amino acids 14-22, 21-35, 45-59, 49-57, 49-63, 105-190, 177-220 or
10 193-208 of UL49 (SEQ ID NO: 3), or amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 (SEQ ID NO: 1).
16. The recombinant virus of claim 15 which is a vaccinia virus, canary pox virus or adenovirus.
17. A pharmaceutical composition comprising the virus of claim 15 and a
15 pharmaceutically acceptable carrier.
18. The pharmaceutical composition of claim 17, further comprising an adjuvant.
19. A method of producing immune cells directed against HSV comprising contacting an immune cell with an antigen-presenting cell, wherein the antigen-presenting cell is modified to present an epitope included in a UL48, UL49 or ICP0
20 protein.
20. The method of claim 19, wherein the epitope comprises amino acids 185-197, 209-221, 288-307 or 430-449 of UL48 (SEQ ID NO: 2), amino acids 14-22, 21-35, 45-59, 49-57, 49-63, 105-190, 177-220 or 193-208 of UL49 (SEQ ID NO: 3), or amino acids 92-101, 92-105, 288-307 or 743-751 of ICP0 (SEQ ID NO: 1).

21. The method of claim 19, wherein the immune cell is a T cell.
22. The method of claim 21, wherein the T cell is a CD4+ or CD8+ T cell.
23. An immune cell produced by the method of claim 19.
24. A method of killing an HSV infected cell comprising contacting an HSV infected
5 cell with the immune cell of claim 23.
25. A method of inhibiting HSV replication comprising contacting a herpes simplex virus with the immune cell of claim 23.
26. A method of enhancing secretion of antiviral or immunomodulatory lymphokines comprising contacting an HSV infected cell with the immune cell of claim
10 23.
27. A method of enhancing production of HSV-specific antibody comprising contacting an HSV infected cell in a subject with the immune cell of claim 23.
28. A method of enhancing proliferation of HSV-specific T cells comprising contacting the HSV-specific T cells with an isolated polypeptide that comprises an
15 epitope included in a UL48, UL49 or ICP0 protein.
29. A method of inducing an immune response to an HSV infection in a subject comprising administering the composition of claim 1 to the subject.
30. A method of inducing an immune response to an HSV infection in a subject comprising administering the composition of claim 11 to the subject.
- 20 31. A method of treating or preventing an HSV infection in a subject comprising administering the composition of claim 1 to the subject.
32. A method of treating or preventing an HSV infection in a subject comprising administering the composition of claim 11 to the subject.

33. A method of treating or preventing an HSV infection in a subject comprising administering an antigen-presenting cell modified to present an epitope included in a UL48, UL49 or ICP0 protein to the subject.

FIG. 1A

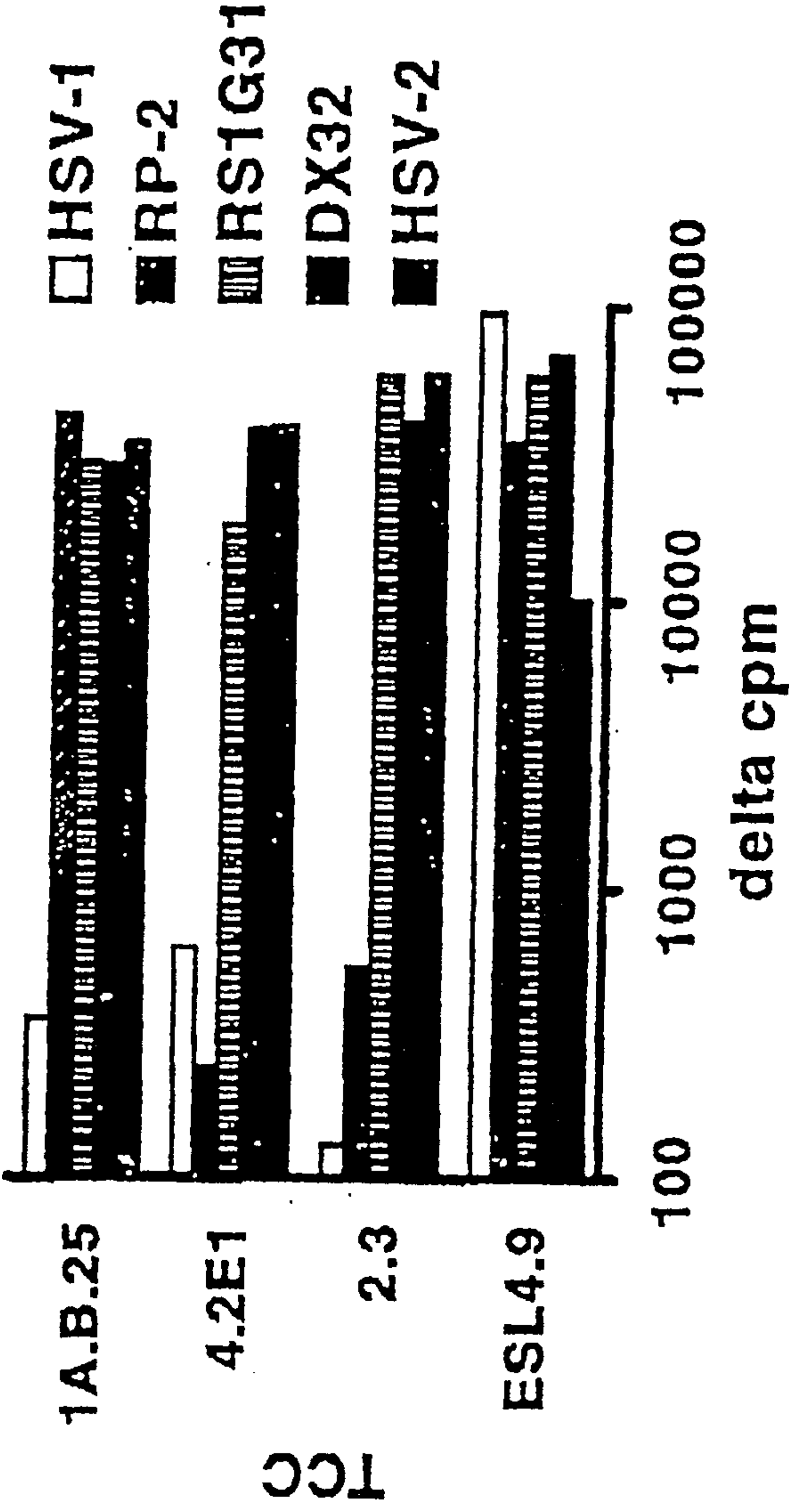
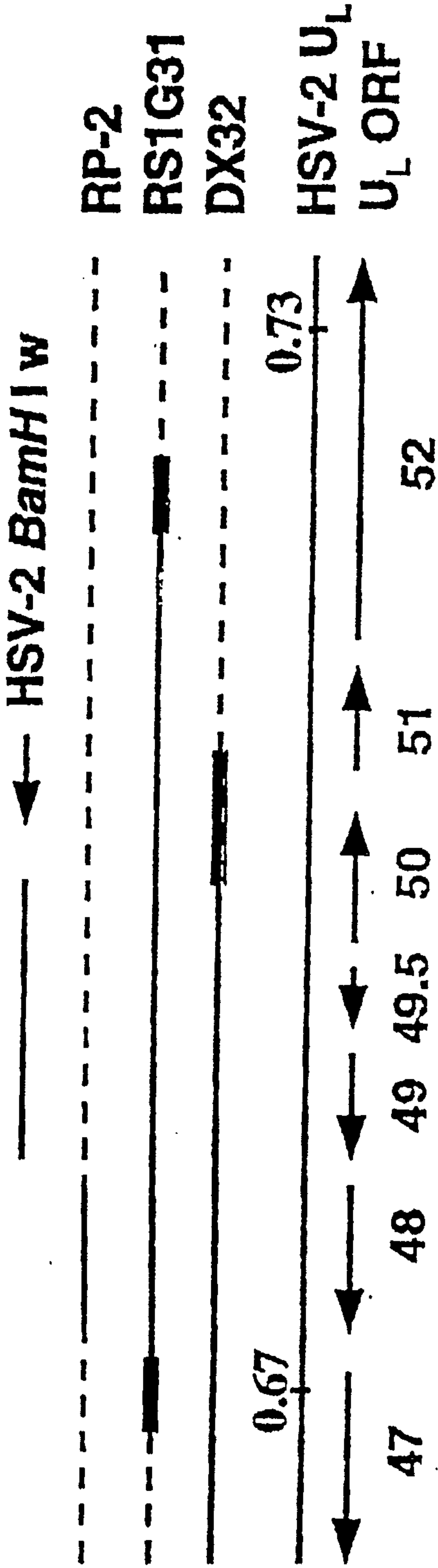


FIG. 1B

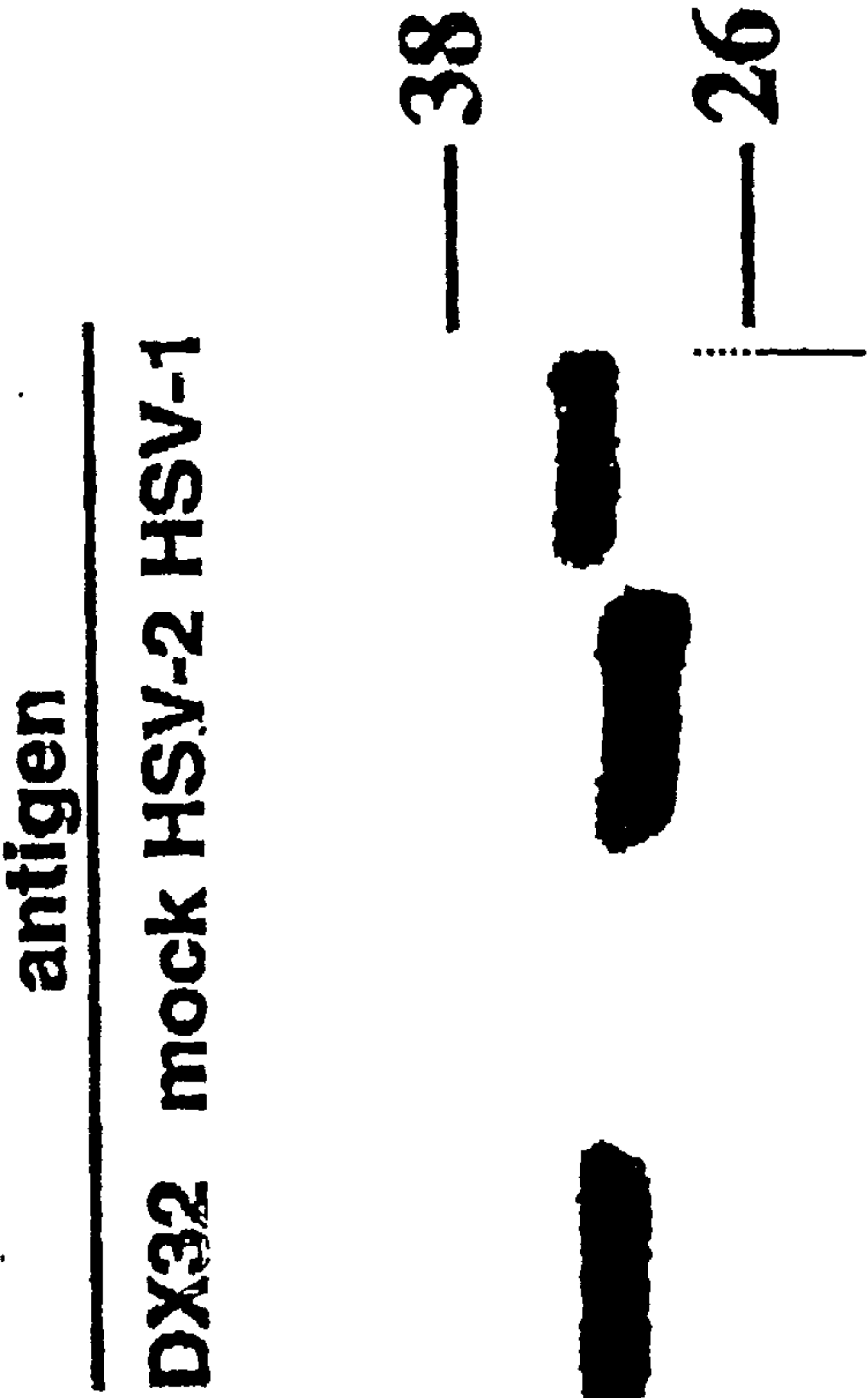
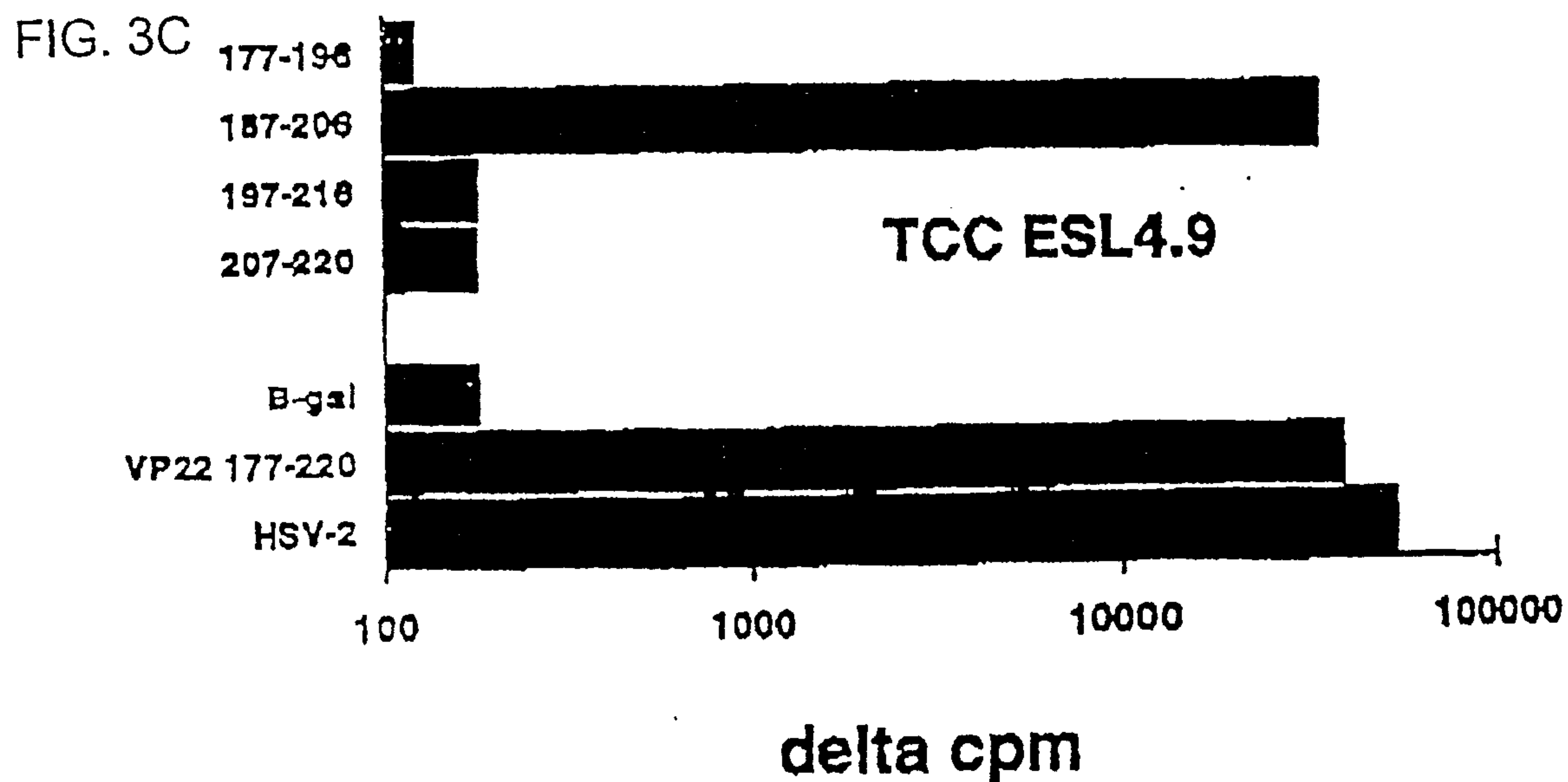
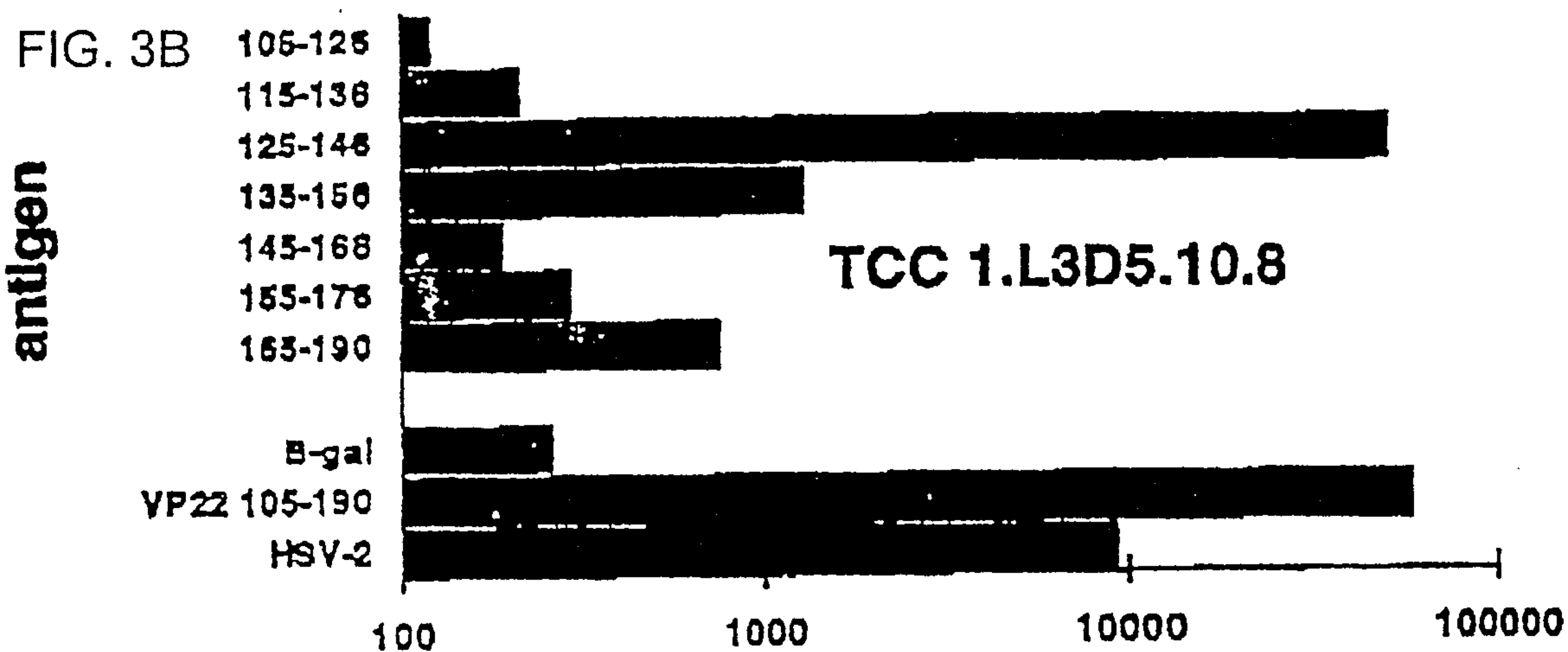
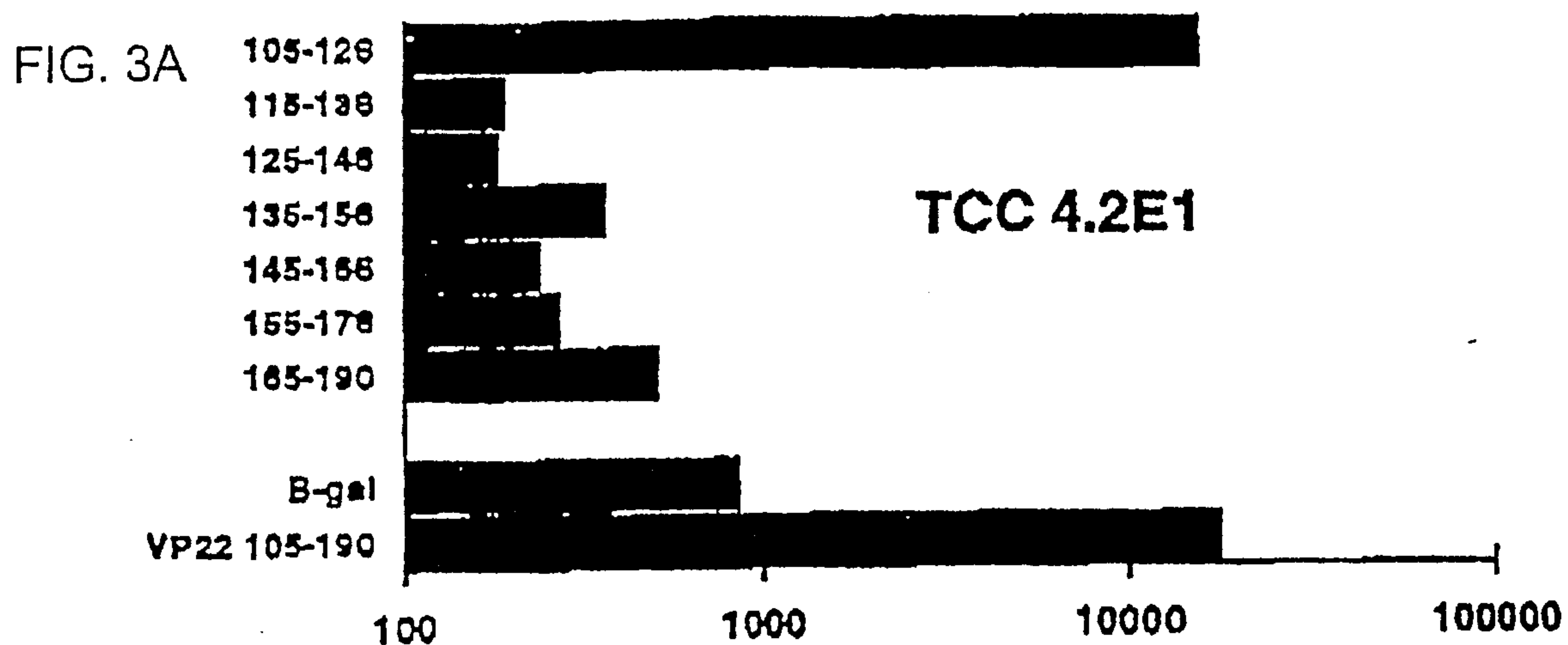


FIG. 2

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4/15

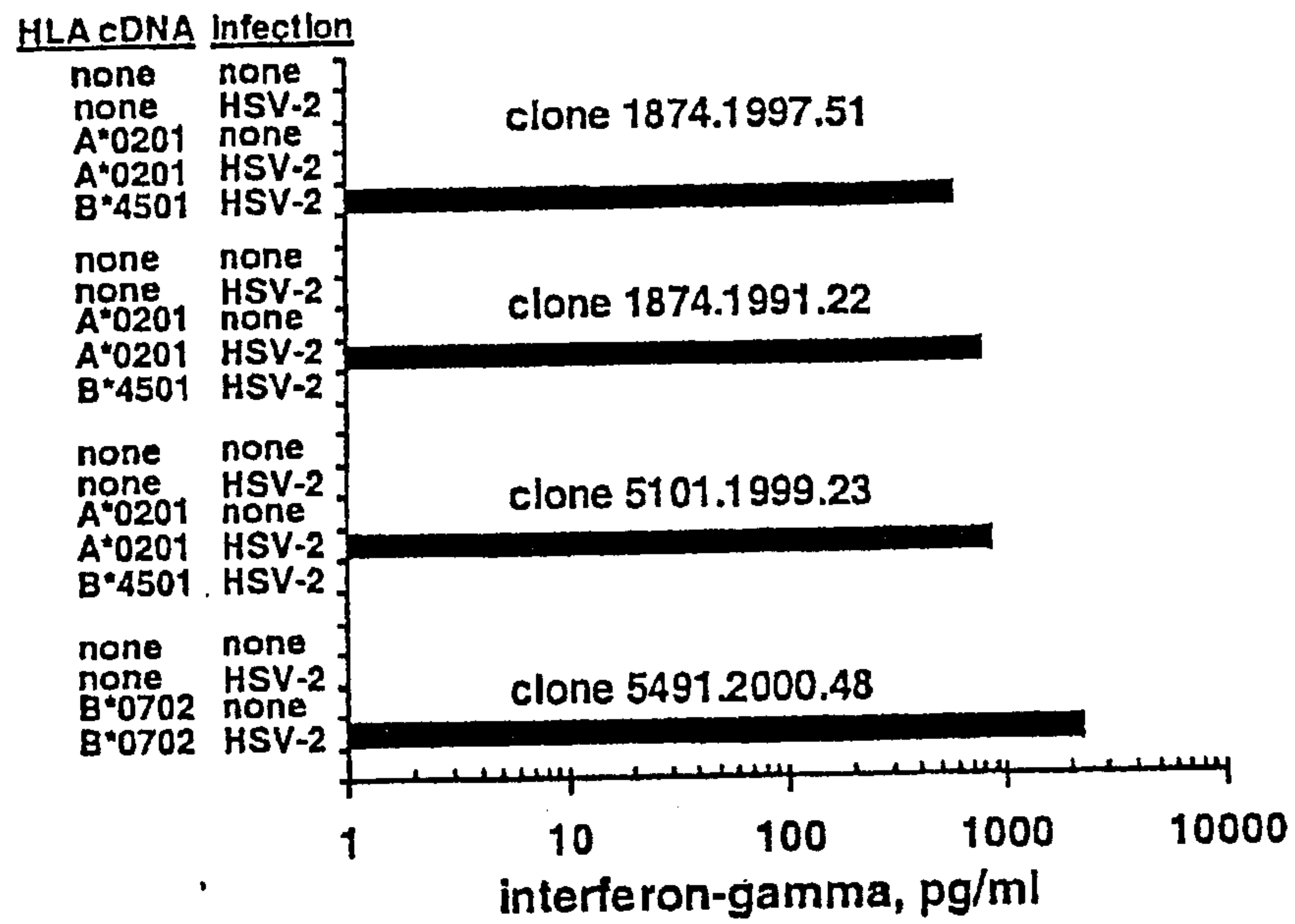


FIG. 4

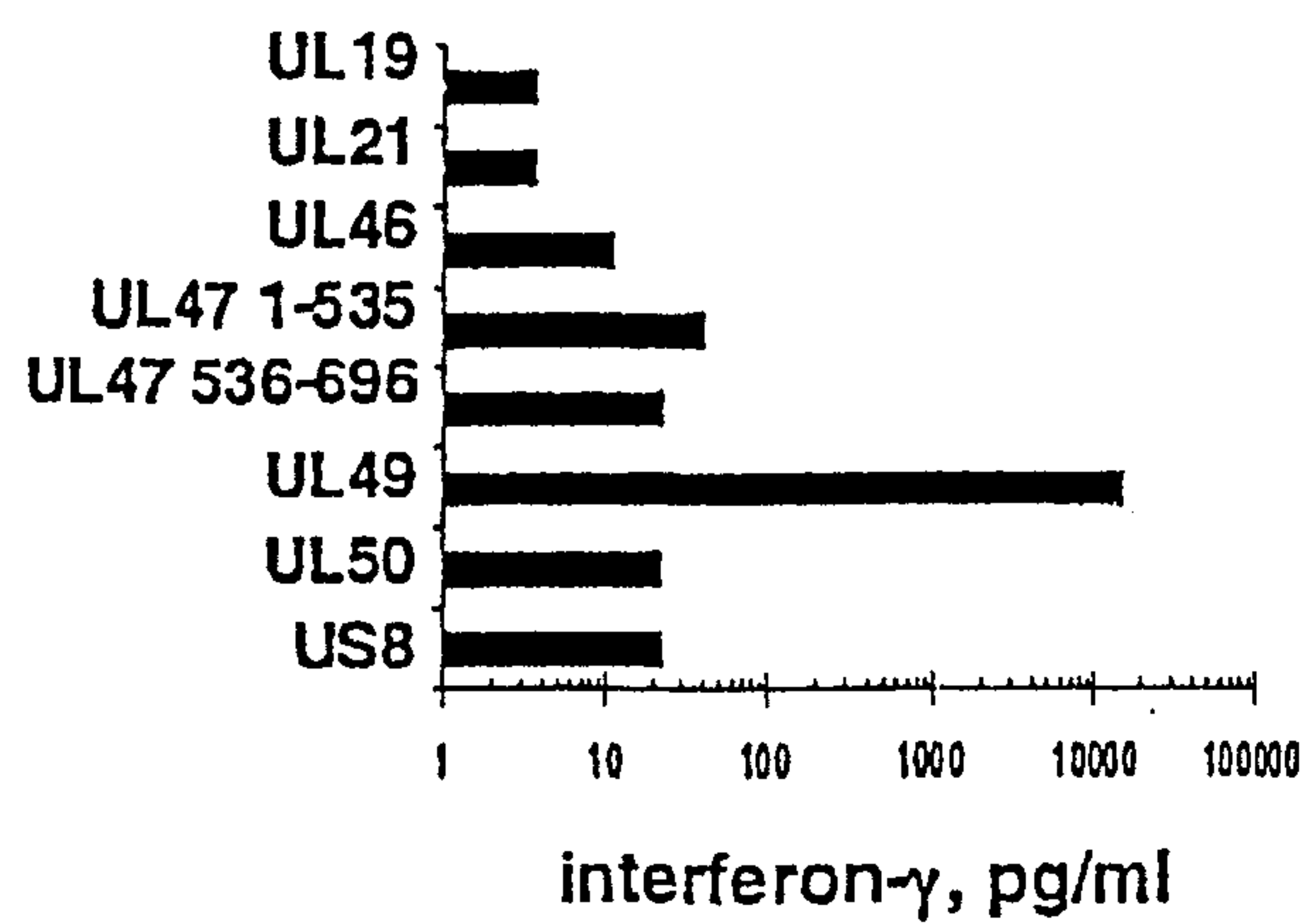


FIG. 5

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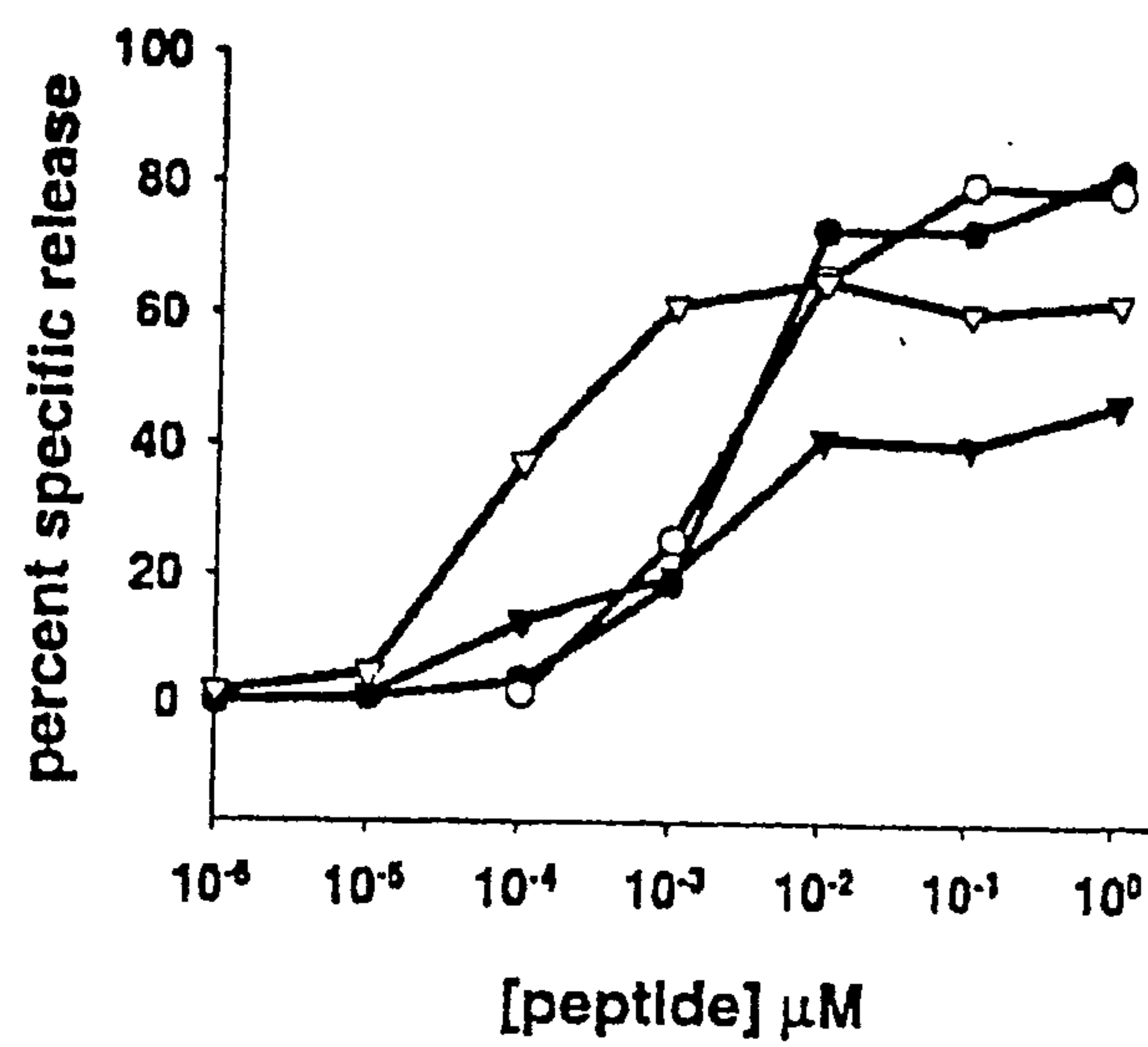


FIG. 6

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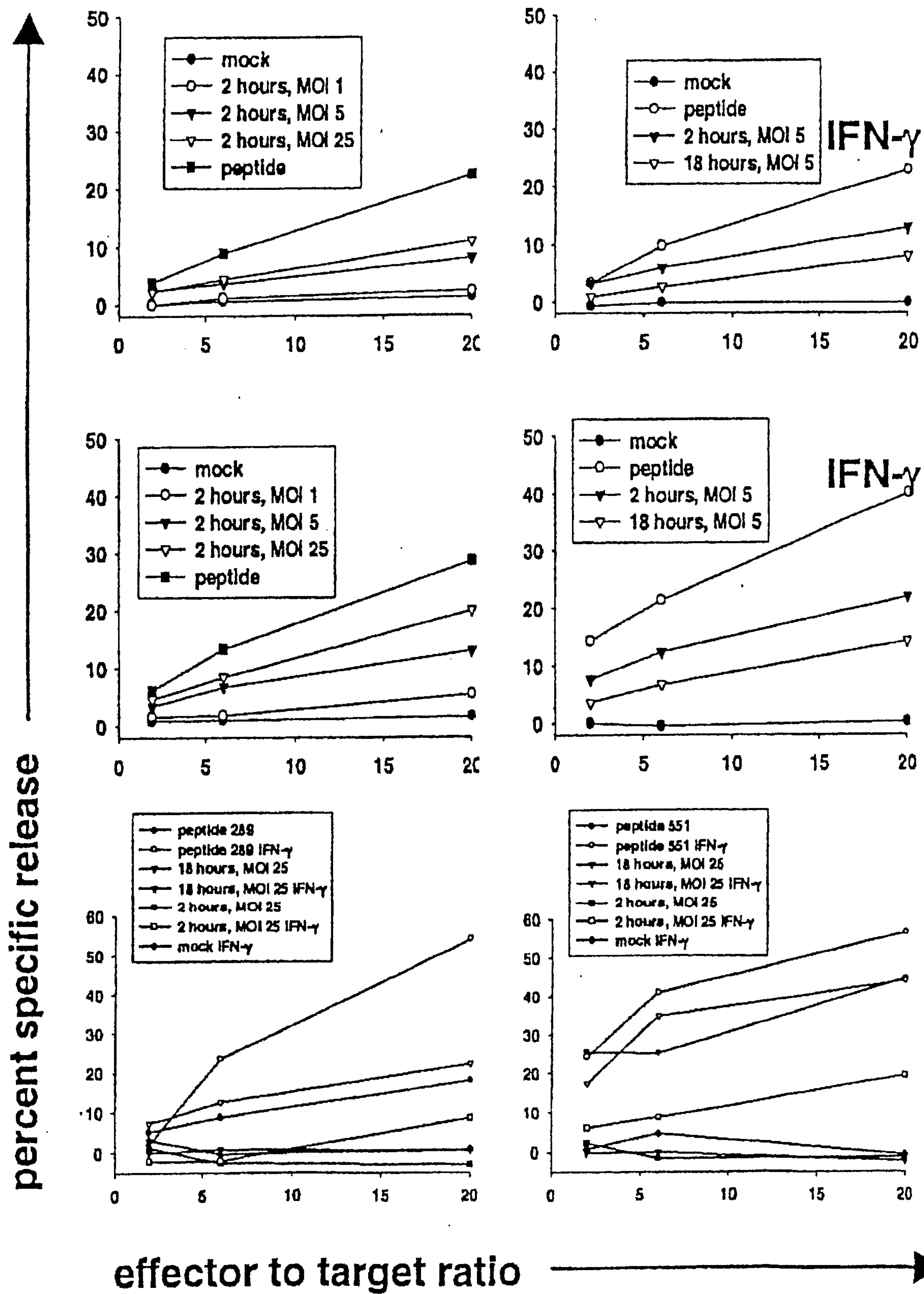


FIG. 7

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FIG. 8C

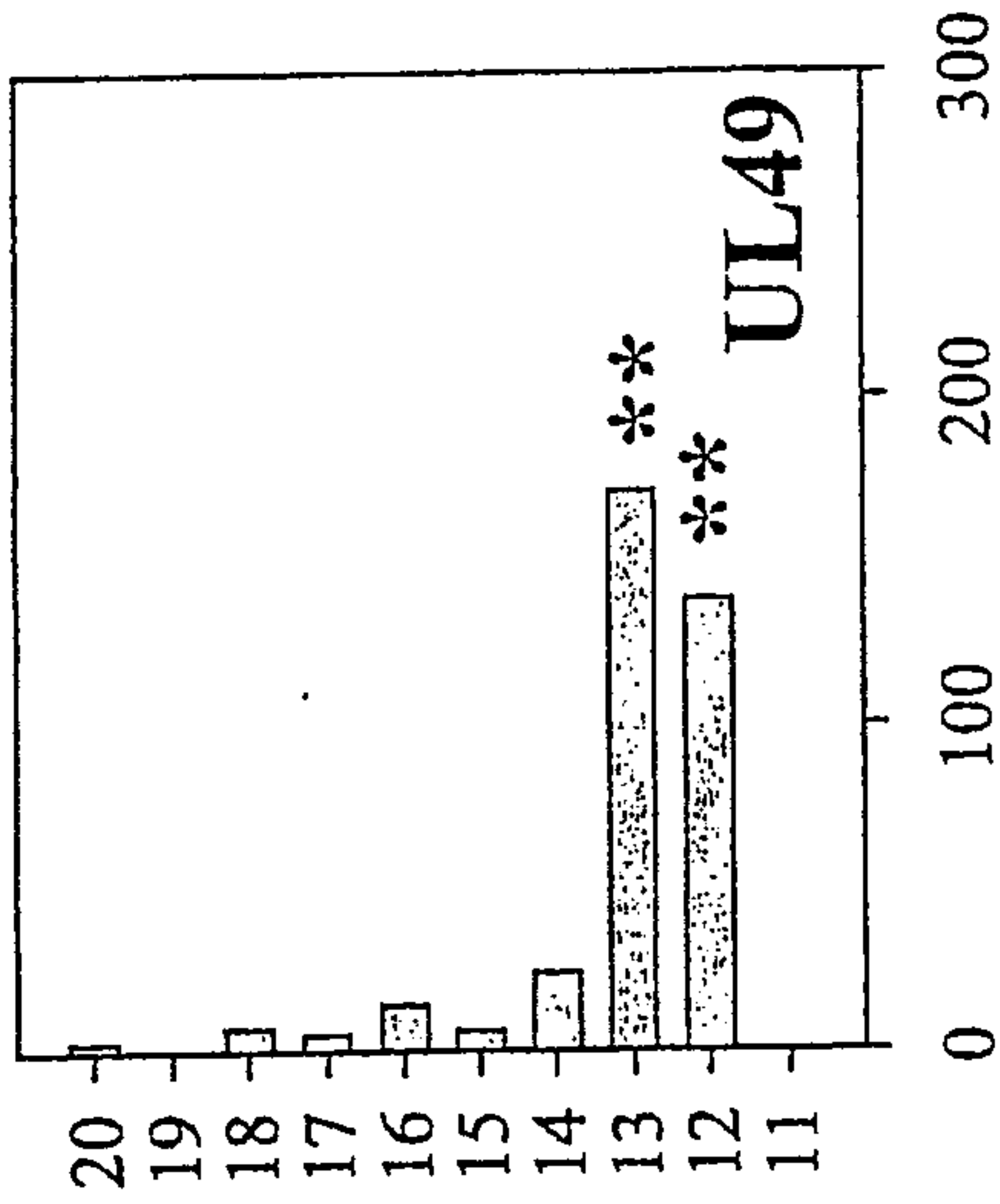


FIG. 8B

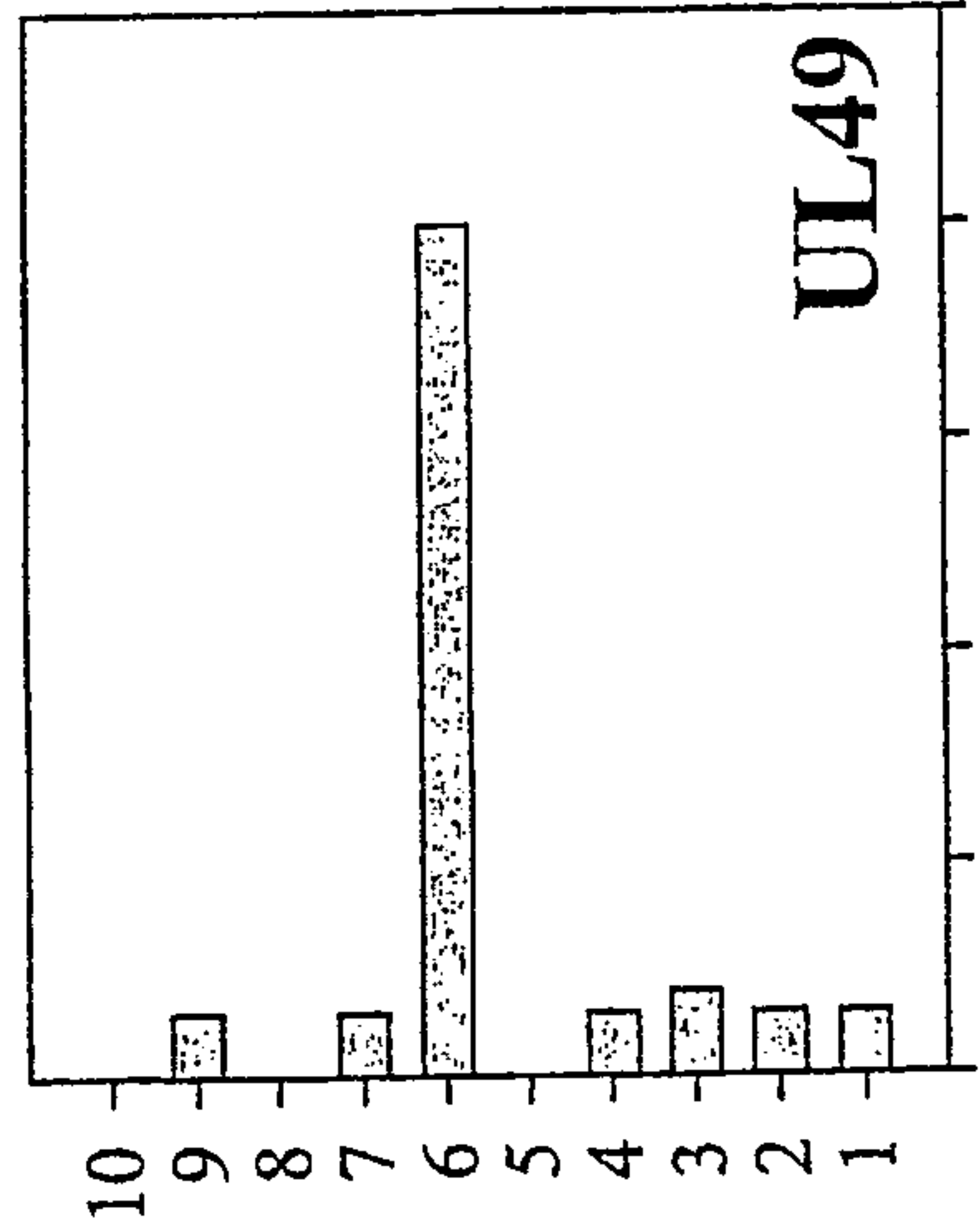
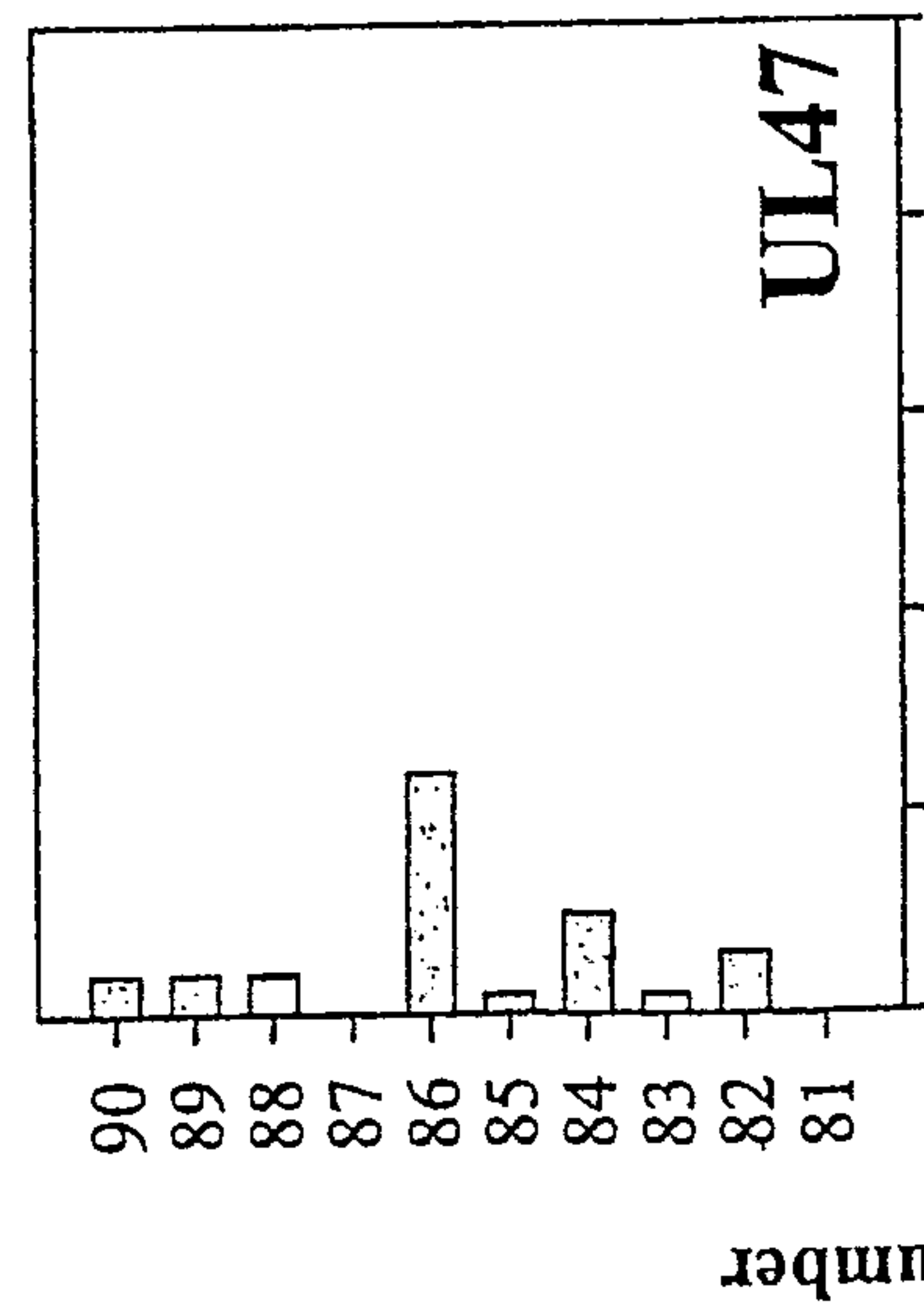
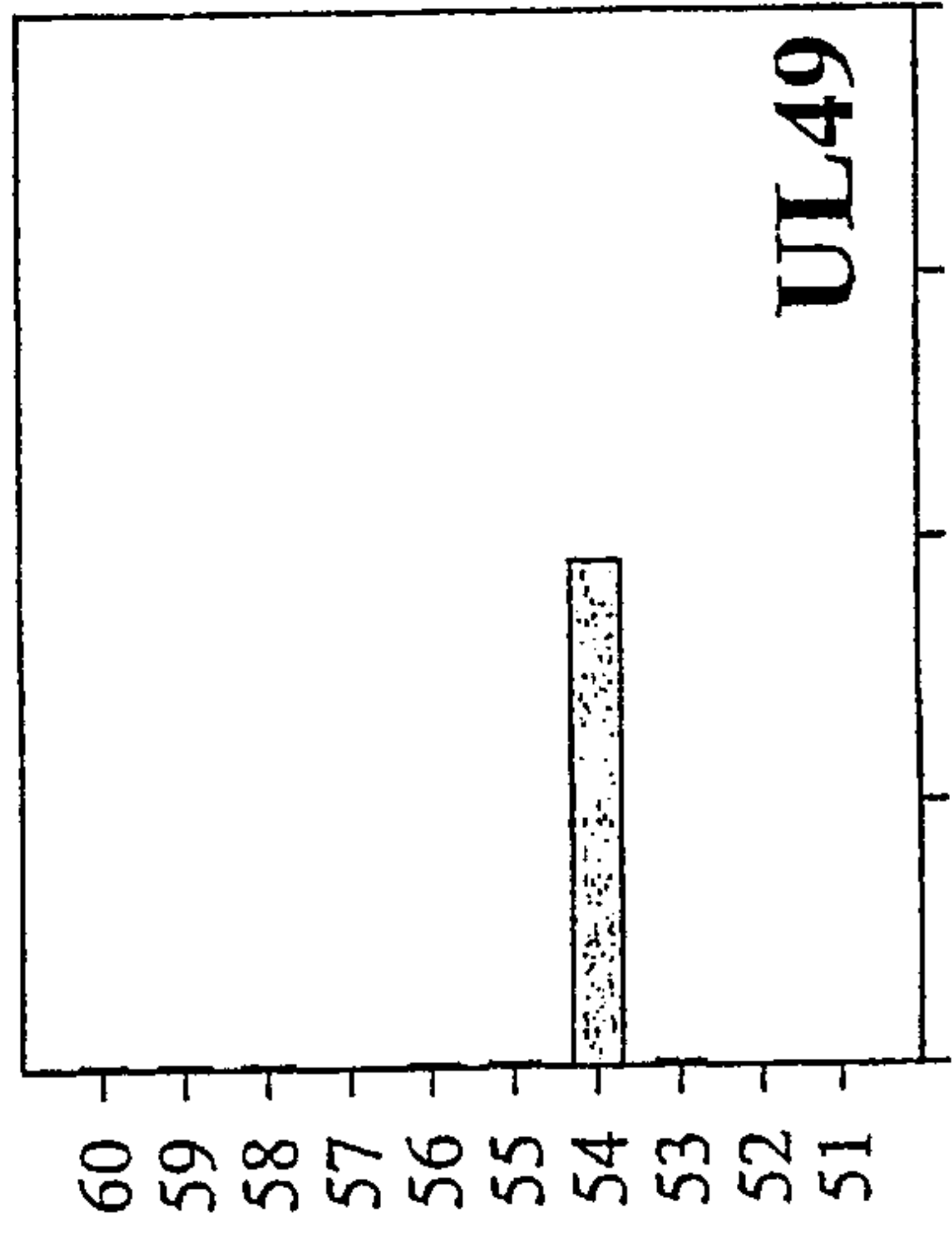


FIG. 8A



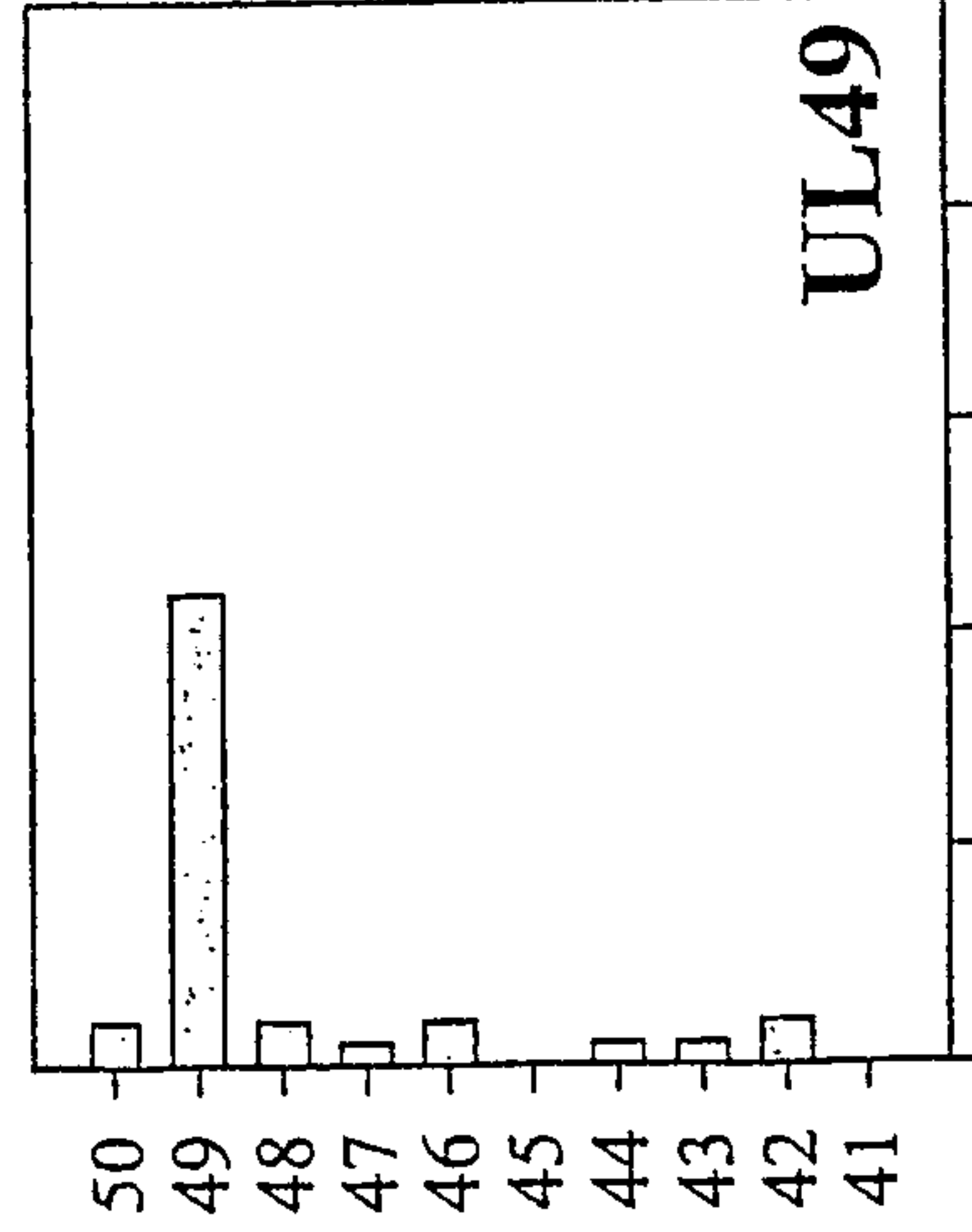
** UL49/49-57 epitope
B*0702-restricted

FIG. 8E



ELISPOTs per 2 x 10e5 CD8's

FIG. 8D



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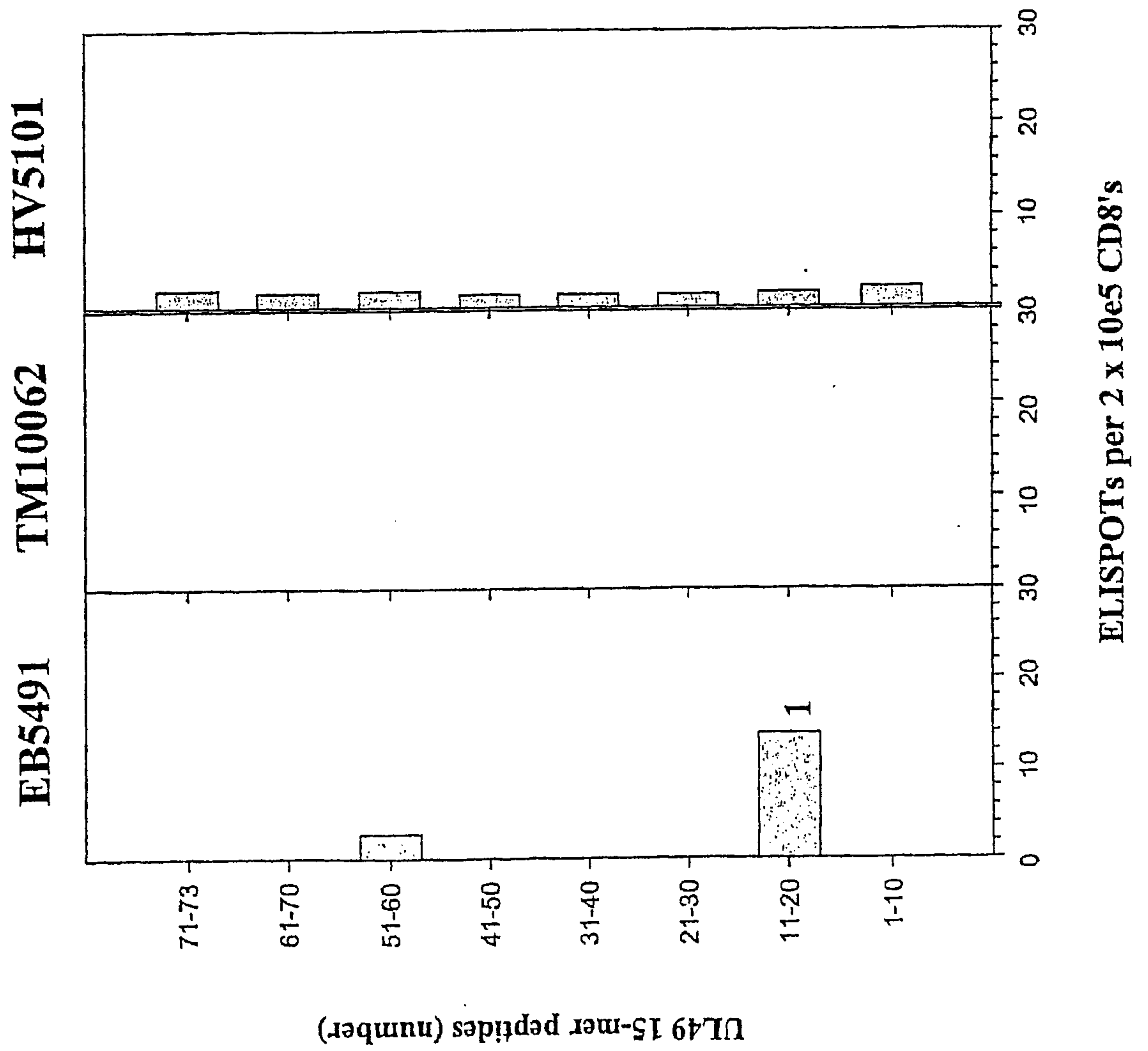


FIG. 9

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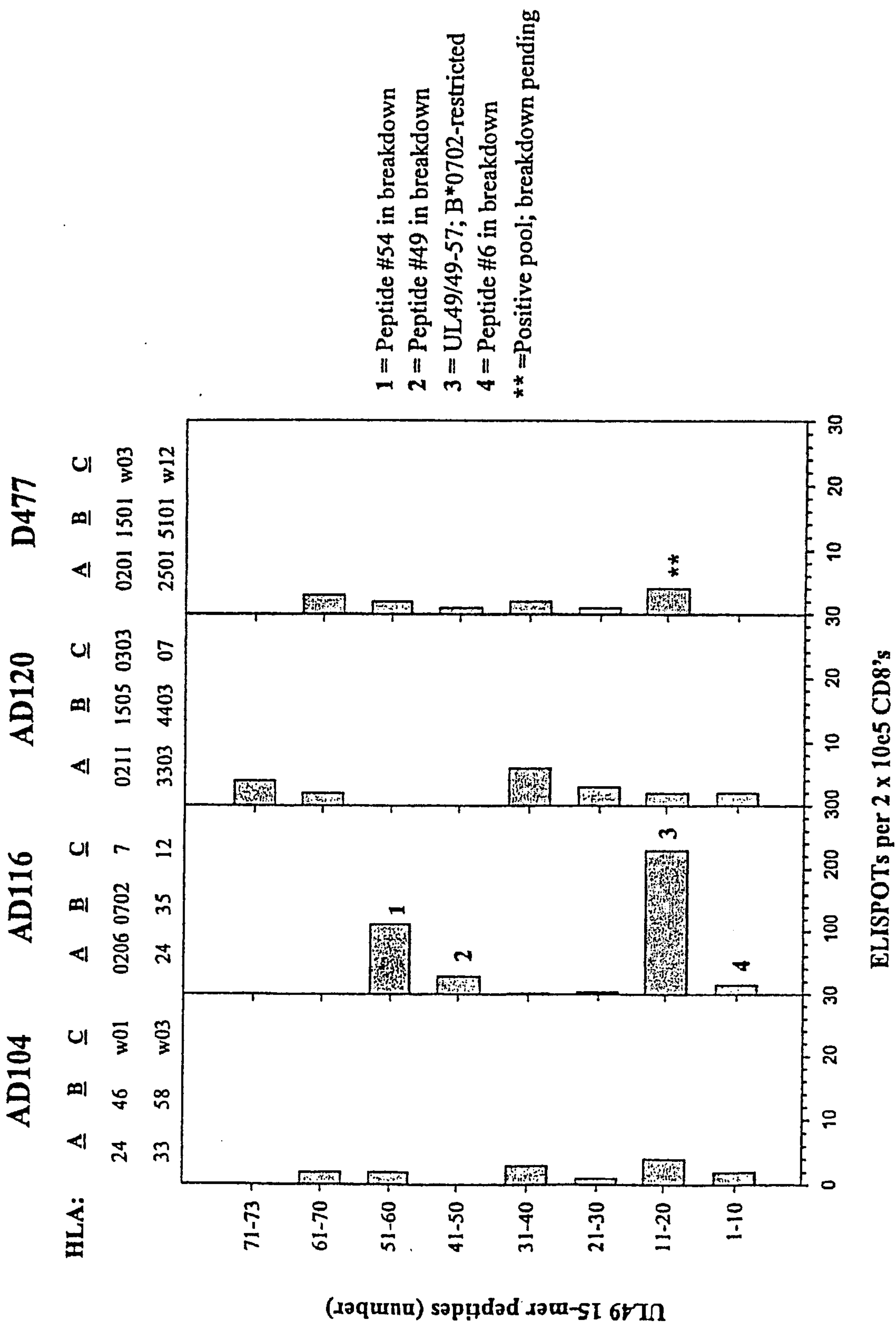


FIG. 10

10/15

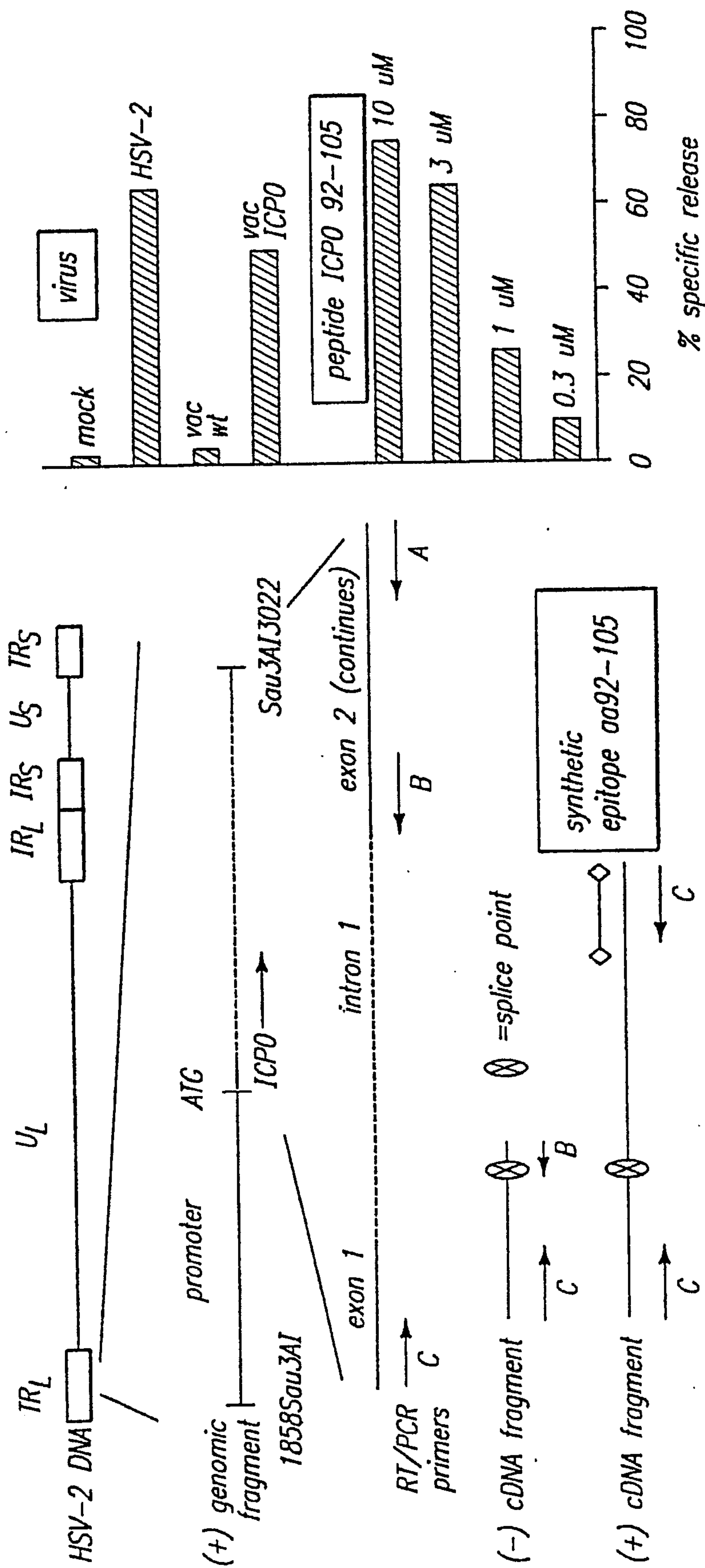


FIG. 11

FIG. 12

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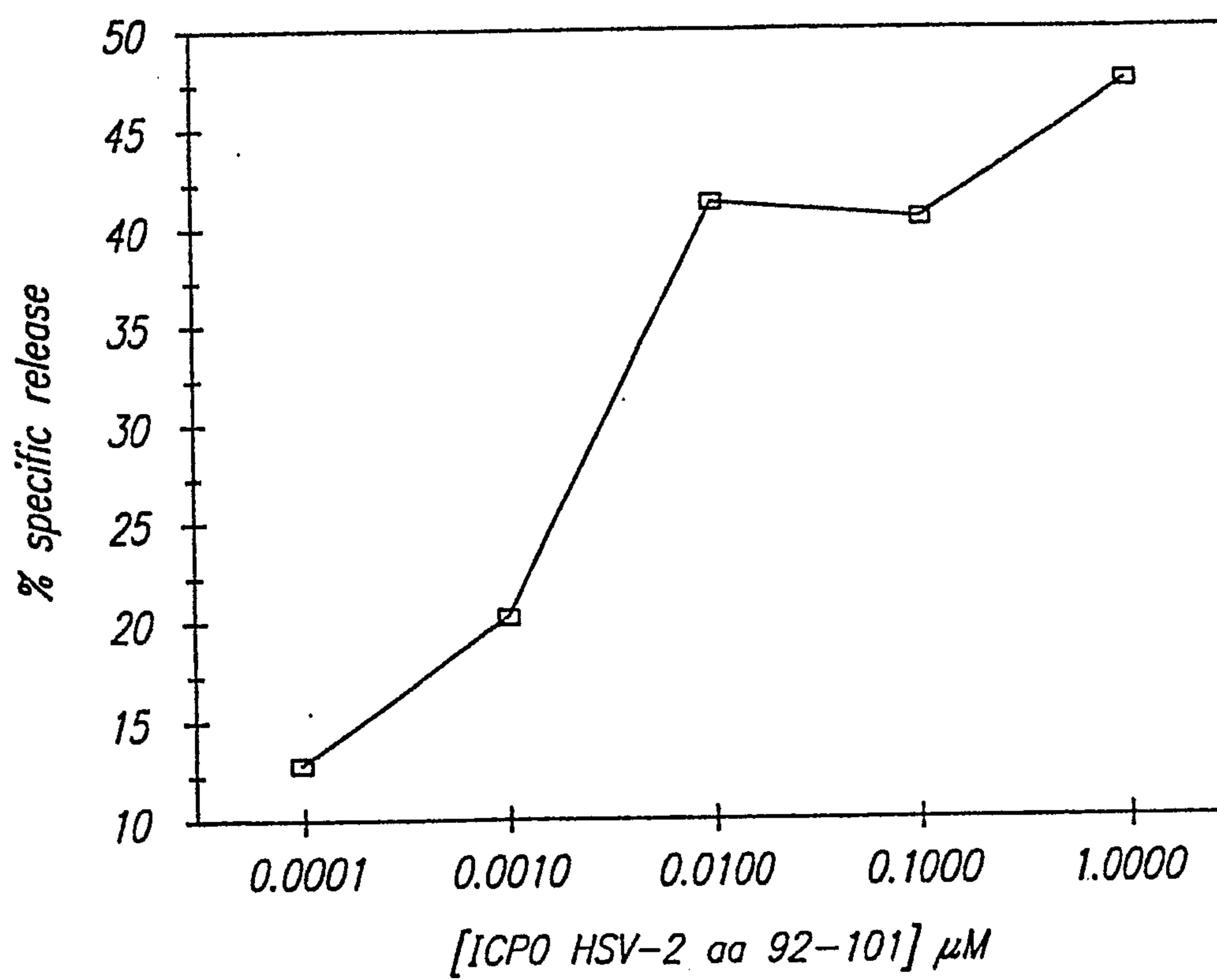


FIG. 13

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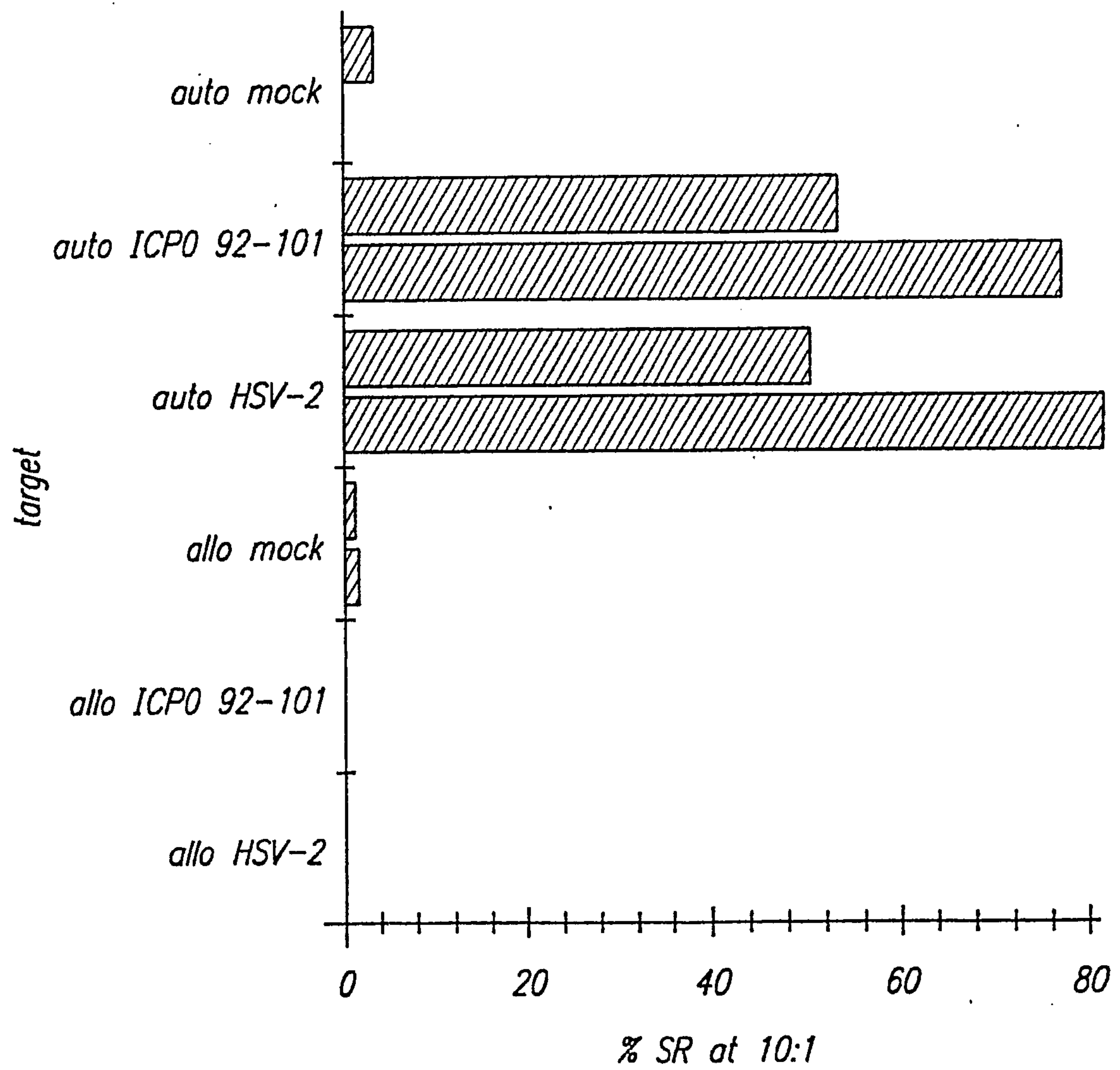


FIG. 14

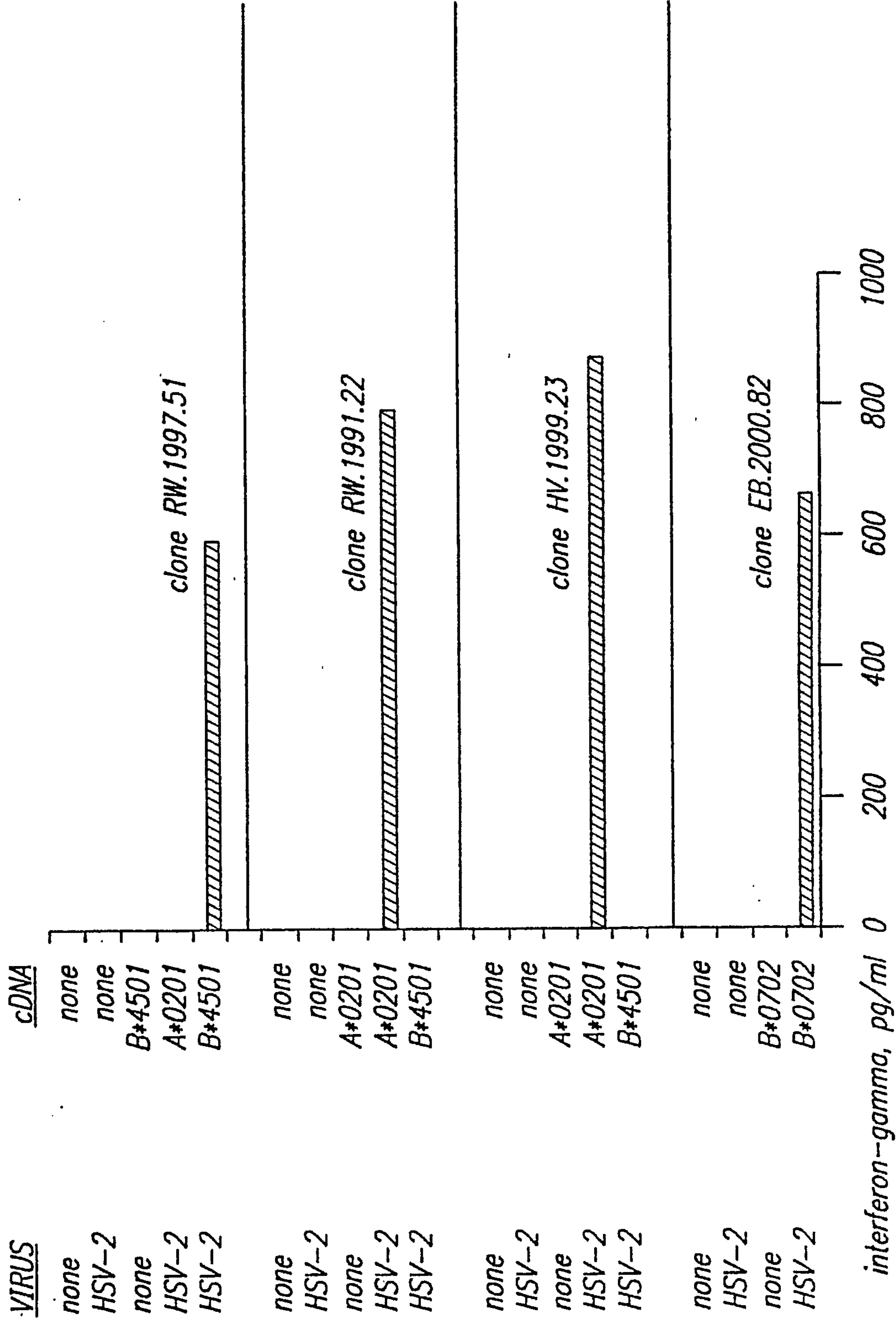


FIG. 15

14/15

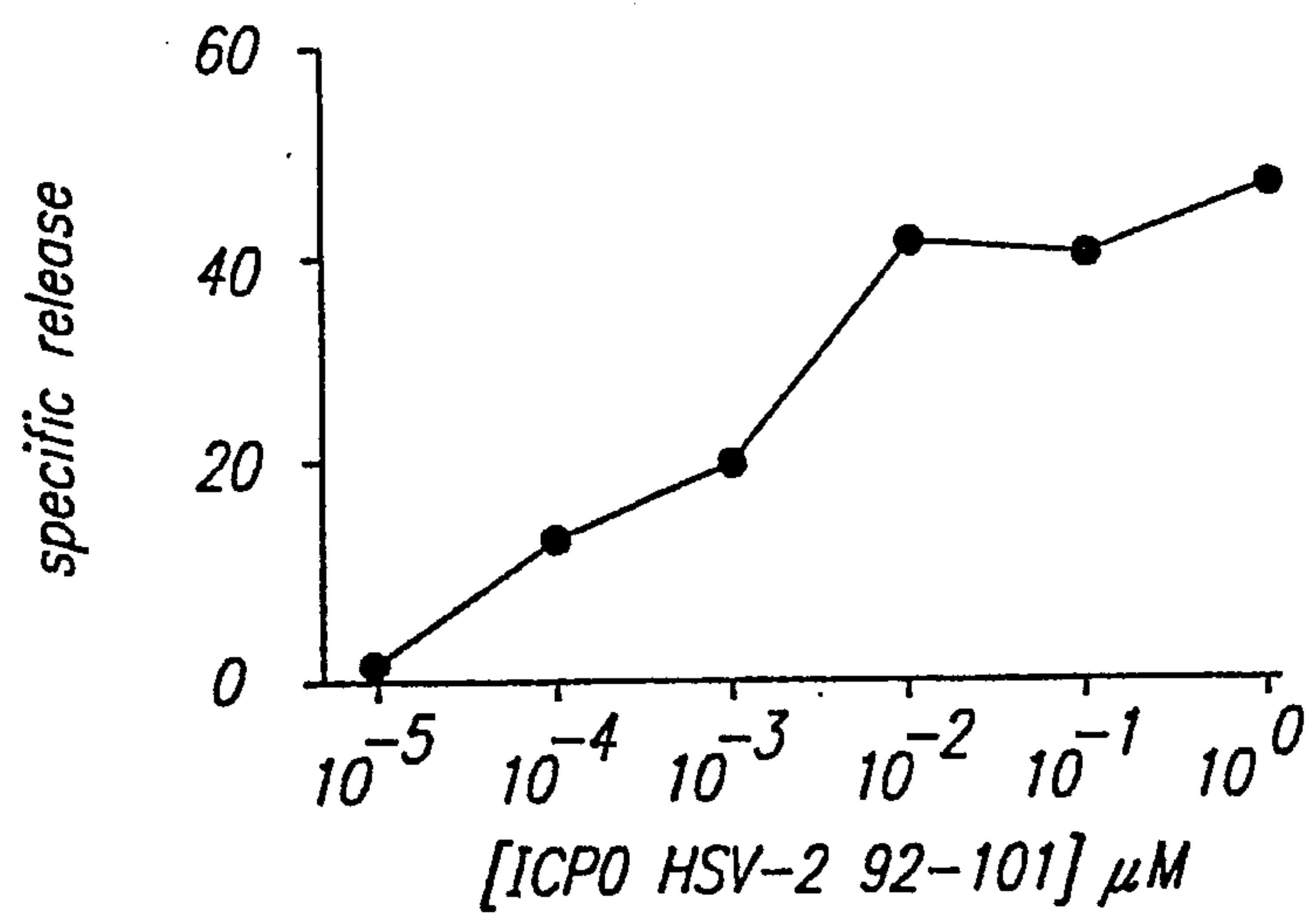


FIG. 16

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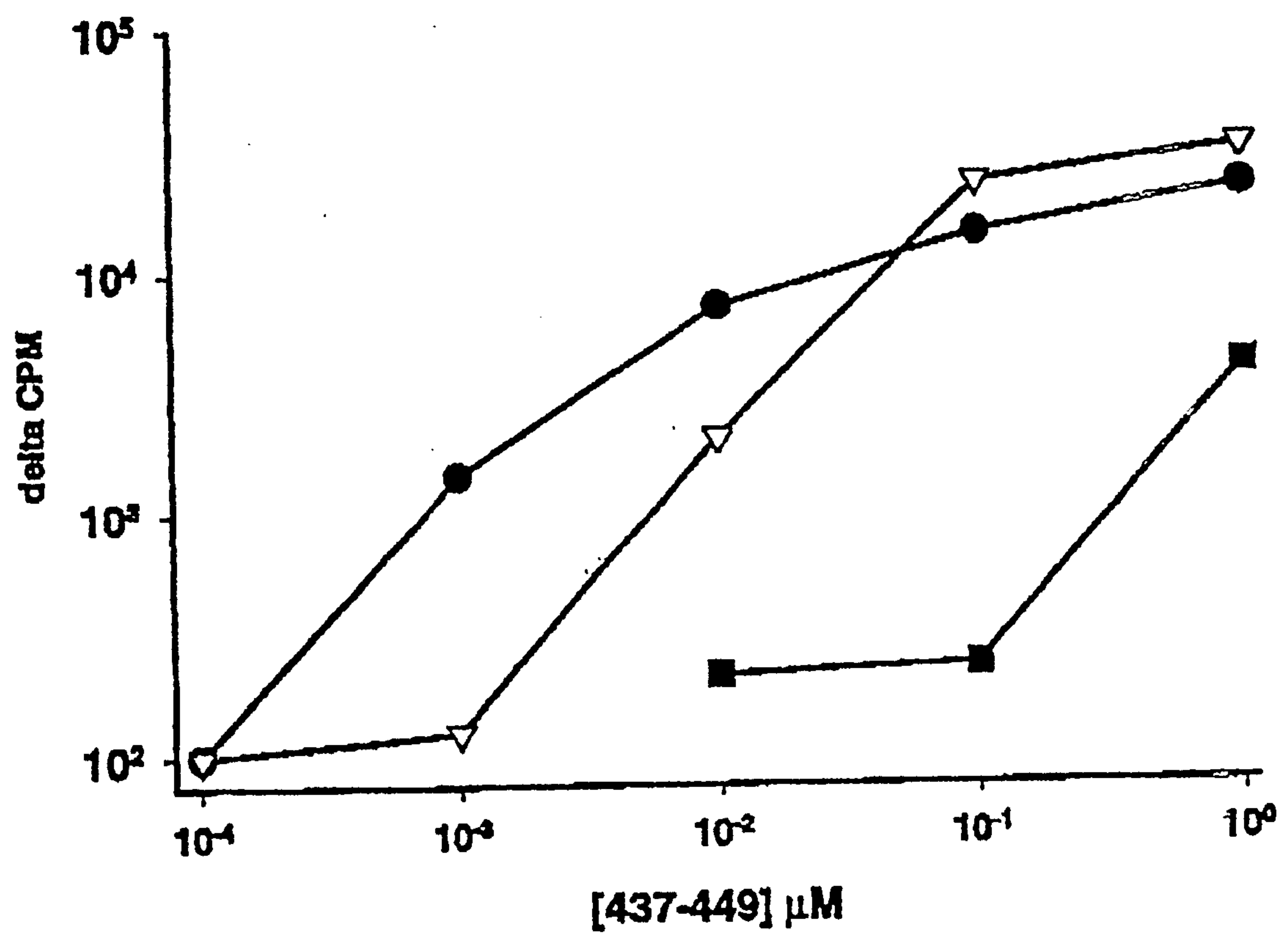
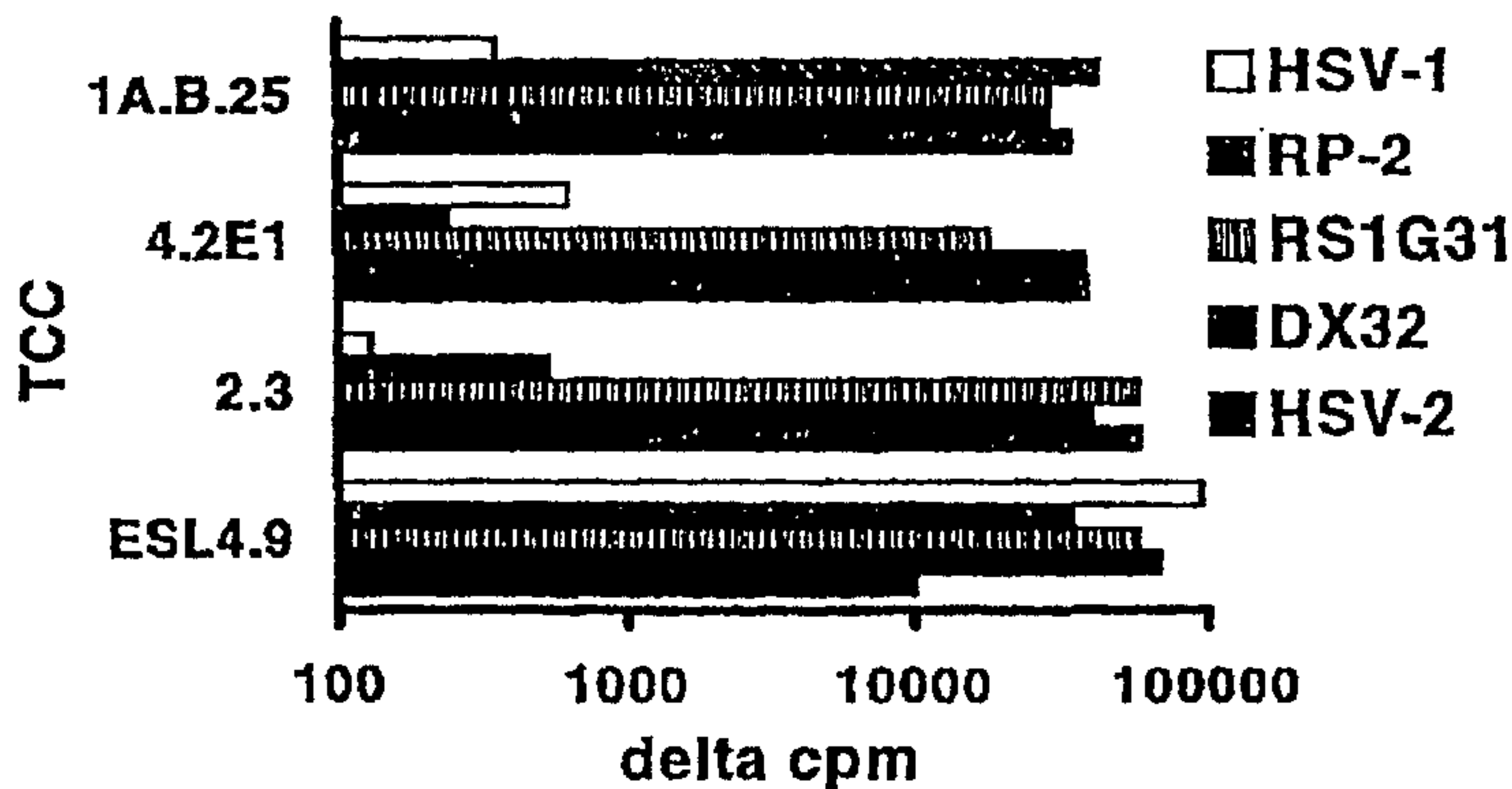
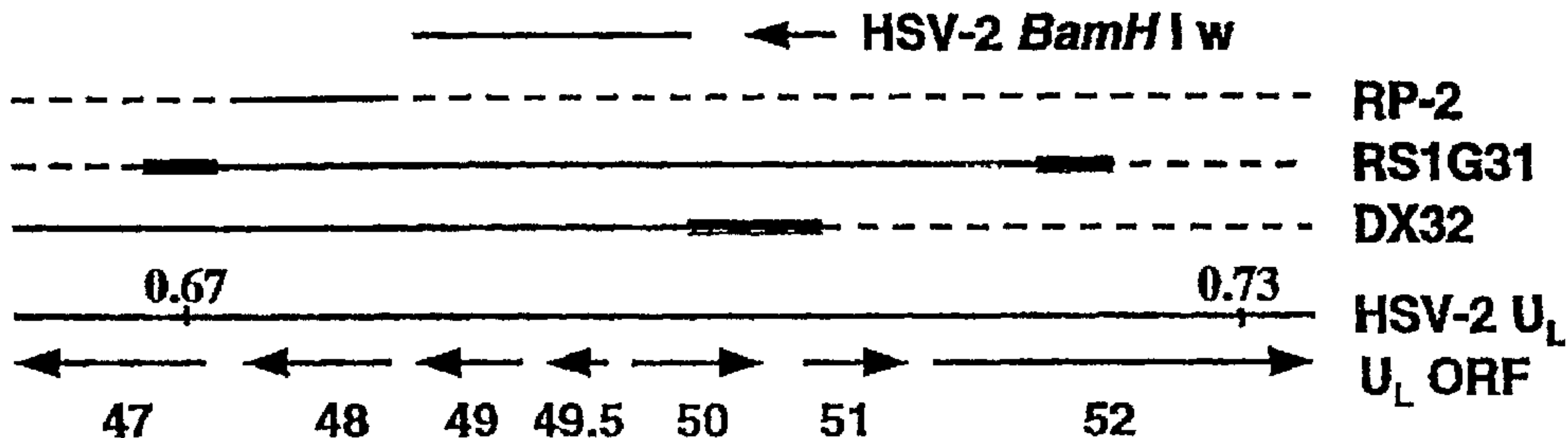


FIG. 17

A



B