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(72) Inventeurs/Inventors:
SCHLOM, JEFFREY, US;
KANTOR, JUDITH, US;
KUFE, DONALD, US;
PANICALI, DENNIS, US;
GRITZ, LINDA, US

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(54) Titre : POXVIRUS RECOMBINE DESTINE A UNE IMMUNISATION CONTRE L'ANTIGENE ASSOCIE AUX
TUMEURS MUC1

(54) Title: RECOMBINANT POX VIRUS FOR IMMUNIZATION AGAINST MUC1 TUMOR-ASSOCIATED ANTIGEN

(57) **Abrégé/Abstract:**

Recombinant pox viruses capable of expressing an immunogenic fragment of the MUC1 tumor-associated antigen are disclosed. The recombinant viruses can be used as vaccines to prevent the establishment of or treat tumors or pre-tumorous cells expressing the MUC1 tumor-associated antigen. The vaccines can be provided as an admixture comprising: (1) a recombinant pox virus encoding the immunogenic fragment of the MUC1 tumor-associated antigen, and (2) a recombinant pox virus encoding a T-cell co-stimulatory factor. The vaccine admixture can be used, e.g., to prevent establishment of tumors or pre-tumorous cells expressing the MUC1 tumor-associated antigen. The MUC1 specific cytotoxic T-cells can be isolated and expanded and used in a method for treating a host having a tumor expressing MUC1 positive tumor cells.



(73) **Propriétaires(suite)/Owners(continued):**

THE GOVERNMENT OF THE UNITED STATES OF AMERICA, REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES, US; DANA-FARBER CANCER INSTITUTE, INC., US

(74) **Agent:** NORTON ROSE OR S.E.N.C.R.L., S.R.L./LLP

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(71) Applicants (for all designated States except US): THERION BIOLOGICS CORPORATION [US/US]; 76 Rogers Street, Cambridge, MA 02142 (US). THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): SCHLOM, Jeffrey [US/US]; 10525 Tyler Terrace, Potomac, MD 20854 (US). KANTOR, Judith [US/US]; 1096 Larkspur Terrace, Rockville, MD 20850 (US). KUFE, Donald [US/US]; 179 Grove Street, Wellesley, MA 02181 (US). PANICALI, Dennis [US/US]; 114 Nonset Path, Acton, MA 01720 (US).

GRITZ, Linda [US/US]; 3 Emerson Street, Somerville, MA 02143 (US).

(74) Agents: EISENSTEIN, Ronald, I. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109-4280 (US).

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(57) Abstract

Recombinant pox viruses capable of expressing an immunogenic fragment of the MUC1 tumor-associated antigen are disclosed. The recombinant viruses can be used as vaccines to prevent the establishment of or treat tumors or pre-tumorous cells expressing the MUC1 tumor-associated antigen. The vaccines can be provided as an admixture comprising: (1) a recombinant pox virus encoding the immunogenic fragment of the MUC1 tumor-associated antigen, and (2) a recombinant pox virus encoding a T-cell co-stimulatory factor. The vaccine admixture can be used, e.g., to prevent establishment of tumors or pre-tumorous cells expressing the MUC1 tumor-associated antigen. The MUC1 specific cytotoxic T-cells can be isolated and expanded and used in a method for treating a host having a tumor expressing MUC1 positive tumor cells.

RECOMBINANT POX VIRUS FOR IMMUNIZATION AGAINST MUC1 TUMOR-ASSOCIATED ANTIGEN

BACKGROUND OF THE INVENTION

5 The immunotherapeutic approach to the treatment of cancer is based on the observation that human tumor cells express a variety of tumor-associated antigens (TAAs) that are not typically expressed in normal tissues. These antigens, which include viral tumor antigens, cellular oncogene proteins, and tumor-associated differentiation antigens, can serve
10 as targets for the host immune system and elicit responses which result in tumor destruction. This immune response is mediated primarily by lymphocytes; T cells in general and class I MHC-restricted cytotoxic T lymphocytes in particular play a central role in tumor rejection. Hellstrom, K.E., et al., (1969) *Adv. Cancer Res.* 12:167-223; Greenberg, P.D. (1991) in
15 *Advances in Immunology*, vol.49 (Dixon, D.J., ed.), pp.281-355, Academic Press, Inc., Orlando, FL. Unfortunately, as evidenced by the high incidence of cancer in the population, the immune response to neoplastic cells often fails to eliminate tumors. The goal of active cancer immunotherapy is the augmentation of anti-tumor responses, particularly T cell responses, in
20 order to effect complete tumor destruction.

Most attempts at active immunization against cancer antigens have involved whole tumor cells or tumor fragments. However, the cloning of TAAs recognized by CD8 + T cells has opened new possibilities for the immunotherapy of cancer based on the use of recombinant or synthetic
25 anti-cancer vaccines. Boon, T., et al., (1994) *Annu. Rev. Immunol.* 12:337-365; Brithcard, V., et al., (1993) *J. Exp. Med.* 178:489-495; Cox, A.L., et al., (1994) *Science* 264:716-719; Houghton, A.N. (1994) *J. Exp. Med.* 180:1-4; Pardoll, D.M. (1994) *Nature* 369:357-358; Kawakami, Y., et al., (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:3515-3519; Kawakami, Y., et al., (1994) *Proc. Natl.*
30 *Acad. Sci. U.S.A.* 91:6458-6462.

DF3/MUC1 (MUC1) is a cell surface glycoprotein that is overexpressed in breast, ovarian, and pancreatic tumors. The major extracellular portion of MUC1 is composed of tandem repeat units of 20 amino acids which comprise immunogenic epitopes. The full length major

extracellular MUC1 protein is composed of up to 100 tandem repeat units of 20 amino acids containing O-glycosylation sites which act as a framework for the formation of a highly glycosylated structure, which is highly immunogenic.

- 5 The term "tandem repeat unit" of MUC1 refers to the 20 amino acid repeated sequence of MUC1 (see, e.g., Gendler, S.J., et al (1990) *J. Biol. Chem.* 265:15286-15293).

(SEQ ID NO:1) **G S T A P P A H G V T S A P D T R P A P**

- 10 There is an abnormal glycosylation pattern found in carcinoma cells making the tumor-derived mucin antigenically distinct from normal mucin. Monoclonal antibodies specific for these peptide epitopes as well as their unique sugar side chains can identify >90% of breast tumors.

- See Kufe, D., et al. (1984) *Hybridoma* 223-32; Taylor-Papadimitriou, J., et al. (1994) *Trends Biotechnol.* 12:227-33; Fontenot, J.D., et al. (1993) *Cancer Res.* 53:5386-94; Siddiqui J., et al. (1988) *Proc. Nati. Acad. Sci. U.S.A* 85:2320-3; Merlo et al. (1989) *Cancer Res.* 49:6966-6971; and Abe, M., et al. (1989) *Biochem Biophys Res Commun* 165:644-9.

- Accordingly, using the MUC1 tumor-associated antigen (TAA) has
20 been proposed in developing cancer vaccines, particularly against tumors expressing MUC1. Multiple copies of tandem repeats are required for optimal native conformation and immunogenicity (see Fontenot et al., supra). A comparison of synthetic peptides containing 3, 4, or 5.25 tandem repeats of MUC1 revealed that the 5.25-copy version most closely mimicked
25 the native structure of MUC1 and showed the most anti-mucin reactivity (Kotera et al. (1994) *Cancer Res.* 54:2856-2860). Previous recombinant vaccinia viruses containing the MUC1 gene with numerous tandem repeats were found to be unstable; homologous recombination resulted in deletion of most of the repeats, reducing the efficacy of the vaccine. See, e.g., Acres, R.B., et al. (1993) *J. Immunother.* 14:136-43; Bu, D., et al. (1993) *J. Immunother.* 14:127-35; Hareuveni, M., et al. (1990) *Proc. Nati. Acad. Sci. U.S.A.* 87:9498-502; and Finn O.J. et al. *infra*.
30

 The use of recombinant vaccinia viruses for anti-tumor immunotherapy has been discussed. (Hu, S.L., Hellstrom, I., and Hellstrom

K.E. (1992) in *Vaccines: New Approaches to Immunological Problems* (R.W. Ellis, ed) pp.327-343, Butterworth-Heinemann, Boston.) Anti-tumor responses have been elicited using recombinant pox viruses expressing TAAs such as carcinoembryonic antigen (CEA) and prostate specific antigen (PSA). (Muraro, R., et al., (1985) *Cancer Res.* 45:5769-5780); (Kantor, 3., et al. (1992) *J. Natl. Cancer Inst.* 84:1084-1091); (Robbins, P.F., et al. (1991) *Cancer Res.* 51:3657-3662.) (Kantor, 3., et al. (1992) *Cancer Res.* 52:6917-6925.) No toxicity with these vectors was observed.

In general, viral vaccines are believed to mediate tumor rejection by activating class I MHC-restricted T-cells, particularly cytotoxic T lymphocytes (CTLs). T-cell activation is often potentiated by providing a suitable immunomodulator, for example a T-cell co-stimulatory factor such as those of the B7 gene family. See e.g., Greenberg, P.D. (1991) in *Advances in Immunology*, Vol.49 (Dixon, D.J., ed.), pp. 281-355, Academic Press, Inc., Orlando, FL; Fox B.A. et al. (1990) *J. Biol. Response Mod.* 9:499-511.

It would be useful to have a recombinant pox virus encoding a MUC1 fragment containing a number of tandemly repeated sequences that will generate a cytotoxic T-cell response to MUC1, but which is stable, undergoing minimal excision as a result of homologous recombination in the gene encoding MUC1. It would also be useful to provide the recombinant pox virus in a vaccine format which is capable of potentiating T-cell activity against such tumors, particularly established or pre-existing tumors expressing the MUC1 TAA.

25

SUMMARY OF THE INVENTION

The present invention relates to recombinant pox viruses encoding a MUC1 fragment, vaccines, and methods of using the recombinant pox viruses and vaccines to generate an immune reaction to MUC1 which can be used to prevent or treat tumors expressing MUC1 TAAs.

30

The recombinant pox virus of the present invention contains a gene encoding an immunogenic MUC1 fragment of 5 to 25 tandem repeats of the 20 amino acid unit, preferably 7-15 tandem repeats, more preferably about 7-10 tandem repeats, still more preferably about 10 tandem repeats, which

when expressed, can vaccinate a mammal against tumors or pre-tumorous cells expressing the MUC1 TAA. This MUC1 gene fragment is stable, maintaining the tandem repeat copy number at around 10 copies.

5 In some preferred embodiments the DNA segment encoding the tandem repeats is altered from the native pattern by using alternative codons to reduce homology between the repeats. For example, amino acids typically have two or more codons that will encode the same residue (e.g., glycine is encoded by GGT, GGA, GGG, or GGC). By using alternative codons encoding the same amino acid one can further reduce the possibility
10 of undesired recombination events. Additionally, one can also introduce some conservative amino acid changes into different groups of the tandem repeats to further reduce undesired recombination (e.g., glycine/serine, valine/leucine), taking care not to alter a peptide epitope that would reduce its immunogenicity.

15 The immunogenic "mini-MUC1 fragments" do not undergo significant genetic deletion, thereby improving stability. Moreover, the fragment imparts sufficient immunogenic specificity for MUC1 immunogenicity. The effect can further be enhanced by providing a T-cell co-stimulatory factor such as B7 and/or a cytokine such as interleukin-2 (IL-2), particularly for
20 the treatment of established or pre-existing tumors expressing the MUC1 TAA.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a Western blot showing expression of a MUC1 polypeptide, referred to as mini-MUC1 or miMUC1, from rV-MUC1 infected MC38 cells.
25

Figs. 2A and 2B are graphs showing expression of MUC1 proteins in MC38 cells. Fig. 2B: MC38 cells transduced with a retroviral vector containing the mini-MUC1 gene and stained with the DF3 anti-MUC1 antibody. Fig. 2A: Non-transduced MC38 cells.

30 Fig. 3 is a graph illustrating primary CTL activity following immunization with an admixture of rV-MUC1 and rV-B7.

Fig. 4 is a graph showing prevention of MUC1-positive pulmonary metastases by immunization with rV-MUC1.

Fig. 5 is a graph illustrating treatment of established MUC1-positive pulmonary metastases by immunization with an admixture of rV-MUC1 and rV-B7.

Fig. 6 is a graph illustrating survival of mice immunized with an admixture of rV-MUC1 and rV-B7.

DETAILED DESCRIPTION OF THE INVENTION

Recombinant pox viruses of the present invention encode a mini-MUC1 fragment that can induce an immunogenic response to MUC1, preferably a cytotoxic T cell response, and can thus serve as effective vectors for vaccination against tumors or pre-tumorous cells expressing the MUC1 TAA. Vaccine efficacy can substantially be enhanced by providing an immune modulator such as a T-cell co-stimulatory factor such as B7-1, B7-2 and/or a cytokine such as IL-2. This is preferred in treating established or pre-existing tumors expressing the MUC1 TAA.

A recombinant pox virus of the present invention can be derived from a naturally-occurring or designated wild-type pox virus strain. The pox virus will be a DNA cytoplasmic pox virus which does not integrate into a host cell genome. Exemplary of such pox viruses are suipox (e.g., swine pox), capripox, leporipox, avipox (e.g., fowl pox, canary pox) and orthopox (e.g., vaccinia, ectromelia, rabbit pox). Representative pox viruses can be obtained from the ATTC such as fowlpox (VR-229) and swinepox (VR-363). A particularly preferred pox virus is vaccinia available from the ATCC as the Wyeth Strain (VR-325).

In one preferred embodiment, the recombinant pox viruses of the present invention made from such pox viruses are characterized as being substantially avirulent. That is, it does not cause disease in the desired target cell or tissue. The selected pox virus may have a host range that does not include the target host species, thereby substantially restricting propagation of the virus in the host. For example, swinepox can be used as a pox virus vector in accordance with the present invention when the host is a primate such as a human. Alternatively, a modified strain of the pox virus can be used to confer avirulence in the normal host range of the pox virus.

Exemplary pox viruses for use in accordance with the present invention are suitable orthopox such as vaccinia viruses, avipox such as fowl pox, canary pox and pigeon pox and suipox such as swine pox. Several suitable strains of vaccinia virus are available, e.g., in an attenuated form
5 such as the MVA or Wyeth strain. These vaccinia strains are substantially attenuated in their normal host range (see e.g., Smith, K.A., et al. (1993) *Vaccine* 11:43-53).

A preferred example of a vaccinia virus suitable for making a recombinant vaccinia virus in accordance with the present invention is the
10 Wyeth strain such as the vTBC33 derivative of the Wyeth strain provided in Example 1. A preferred avipox is fowlpox.

An immunogenic MUC1 fragment can be inserted into a suitable pox virus by conventional recombinant techniques to produce the present recombinant pox viruses. For example, as will be explained in more detail
15 in Reference Example 1 and the examples which follow, a DNA donor plasmid vector including a DNA insert encoding an immunogenic MUC1 fragment can be constructed to provide recombination between DNA sequences flanking the insert in the donor plasmid vector and homologous sequences present in the virus. Accordingly, a recombinant virus encoding
20 the immunogenic MUC1 fragment is formed therefrom. Other techniques can be used to make the recombinant virus encoding the immunogenic MUC1 fragment including use of a unique restriction endonuclease site that is naturally present or artificially inserted in the parental viral vector (see
25 e.g., Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982); and U.S. Patent No.5,093,258).

More particularly, the immunogenic MUC1 fragment can be inserted by conventional methods into the DNA donor vector such as those suitable for use in a prokaryote such as *E. coli*. The donor vector will further include viral DNA which is homologous to a segment of pox virus DNA at the site to
30 which insertion of the MUC1 fragment is desired. DNA encoding the immunogenic MUC1 fragment can be inserted into the DNA donor vector adjacent to suitable control elements in the vector such as promoter, enhancer, ribosome binding, and leader sequences. The DNA so inserted into the donor vector is typically positioned to provide flanking viral DNA

(e.g., vaccinia *Hind*III fragments) on both ends of the insert. As stated previously, the flanking viral DNA will generally be homologous to a DNA sequence flanking a region of the pox virus DNA to which insertion is desired. Preferably, the homologous flanking viral DNA sequence will be
5 100% homologous to the region of the pox virus DNA to which insertion is desired. Exemplary DNA donor vectors generally include an origin of replication such as the *E. coli* origin of replication, and a marker such as an antibiotic resistance gene for selection and propagation in a suitable host such as *E. coli*. The resulting DNA donor vector is then propagated by
10 growth within a suitable prokaryotic host cell, isolated and purified if desired.

The DNA donor vector including the immunogenic MUC1 fragment to be inserted into a desired pox virus is generally transfected into a suitable cell culture, e.g., a primate cell line or chick embryo fibroblasts, that is
15 infected with the pox virus. Recombination between homologous DNA in the DNA donor vector and the pox virus genome forms a recombinant pox virus modified by the presence of the immunogenic MUC1 fragment. Preferably, the site of pox virus insertion does not substantially affect the viability of the recombinant pox virus. Viral viability can be readily tested
20 by, e.g., viral plaque assay or a DNA replication assay involving tagging newly synthesized DNA with a detectably-labeled nucleotide (e.g. ³H-thymidine). Typically, viral viability will be assessed by comparing the viability of the recombinant pox virus to that of a control pox virus (i.e., no inserted DNA).

25 As noted above, the immunogenic MUC1 fragment is inserted into a suitable region (insertion region) of a pox virus so that virus viability is not substantially affected. The skilled artisan can readily identify such regions in the pox virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without affecting virus viability
30 of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, it has been found in all pox virus genomes examined (e.g., leporipoxvirus: Upton, et al., *J. Virology*, 60:920 (1986) (shope fibroma virus); capripoxvirus: Gershon, et al., *J. Gen. Virol.*, 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, et al.,

J. Virol., 46:530 (1983) (vaccinia); Esposito, et al., *Virology*, 135:561(1984) (monkeypox and variola virus); Hruby, et al., *PNAS*, 80:3411(1983) (vaccinia); Kilpatrick, et al., *Virology*, 143:399 (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al., *J. Gen. Virol.* 69:1275(1988) (fowlpox);
5 Boyle, et al., *Virology*, 156:355(1987) (fowlpox); Schnitzlein, et al., *J. Virological Methods*, 20:341(1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., *J. Gen. Virol.* 73:3235-3240 (1992)).

In vaccinia, in addition to the TK region, other insertion regions include, for example, HindIII M.

10 In fowlpox, in addition to the TK region, other insertion regions include, for example, BamHI J [Jenkins, et al., *AIDS Research and Human Retroviruses* 7:991-998 (1991)] the *EcoRI-HindIII* fragment, *BamHI* fragment, *EcoRV-HindIII* fragment, *BamHI* fragment and the *HindIII* fragment set forth in EPO Application No. 0 308 220 Al. [Calvert, et al., *J. of Virol.* 67:3069-
15 3076 (1993); Taylor, et al., *Vaccine* 6:497-503 (1988); Spehner, et al., (1990) and Boursnell, et al., *J. Gen. Virol.* 71:621-628 (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region and the HindIIIC region.

In addition to the requirement that the gene be inserted into an
20 insertion region, successful expression of the inserted gene by the modified poxvirus requires the presence of a promoter operably linked to the desired gene, i.e., in the proper relationship to the inserted gene. The promoter must be placed so that it is located upstream from the gene to be expressed. Promoters are well known in the art and can readily be selected depending
25 on the host and the cell type you wish to target. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, or 40K or fowlpox Cl. Artificial pox promoter constructs containing appropriate promoter sequences can also be used. Enhancer elements can also be used in combination to increase the level of expression. Furthermore, the use of
30 inducible promoters, which are also well known in the art, are preferred in some embodiments.

For example, it is possible to make a DNA vector construct in which the promoter is modulated by an external factor or cue, and in turn to control the level of polypeptide being produced by the vectors by activating

that external factor or cue. For example, heat shock proteins are proteins encoded by genes in which the promoter is regulated by temperature. The promoter of the gene which encodes the metal-containing protein metallothionine is responsive to Cd⁺ ions. Incorporation of this promoter or
5 another promoter influenced by external cues also makes it possible to regulate the production of the proteins.

The pox vectors of the present invention contain a DNA fragment encoding a MUC1 fragment, sometimes referred to as mini-MUC. The MUC1 gene fragment will encode a sufficient portion of MUC1 to generate an
10 immune reaction to MUC1, but does not undergo extensive excision as a result of homologous recombination. Preferably, the fragment is approximately 5 to 25 MUC1 tandem repeat units, more preferably between approximately 7 to 15 MUC1 tandem repeat units, and most preferably about 7 to 12 MUC1 tandem repeat units. An especially preferred
15 immunogenic MUC1 fragment is about 10 MUC1 tandem repeat units. Preferred fragments have the human MUC1 DNA sequence. A preferred MUC1 DNA sequence is the human MUC1 cDNA sequence having the repeat units disclosed, e.g., by Gendler et al. *supra*. While the sequence reported by Merlo et al., *supra*, is 10 MUC1 tandem repeat units, a sample
20 based on this was only about 7 tandem repeat units. This sample is more fully described in the examples.

In some preferred embodiments the DNA segment encoding the tandem repeats is altered from the native pattern in such a manner as to reduce duplications of the codons. For example, amino acids typically have
25 two or more codons that will encode the same residue (e.g., glycine is encoded by GGT, GGA, GGG, or GGC). By using other codons encoding the same amino acid one can further reduce the possibility of undesired recombination events. Additionally, one can also introduce some conservative amino acid changes into different groups of the tandem repeats
30 to further reduce undesired recombination (e.g., glycine/serine, valine/leucine), taking care not to alter a peptide epitope that would reduce its immunogenicity.

Preferably, the 60 bp tandem repeat sequence can be altered to minimize nucleotide homology without changing the amino acid sequence.

For example the first tandem repeat in miMUC1 can be left unaltered as follows:

	1	2	3	4	5	6	7	8	9	10
5	GGC	TCC	ACC	GCC	CCC	CCA	GCC	CAC	GGT	GTC
	G	S	T	A	P	P	A	H	G	V
	11	12	13	14	15	16	17	18	19	20
10	ACC	TCG	GCC	CCG	GAC	ACC	AGG	CCG	GCC	CCG
	T	S	A	P	D	T	R	P	A	P

*(Sequence ID NO:2)

15 The second, third, and fourth tandem repeats can then be altered in the third base of threonine codons 3, 11 and 16 using ACG, ACT, and ACA, respectively. These repeats can also be altered in alanine codons 4, 7, 13, and 19, using GCG, GCA, and GCT respectively. Similar third-base alterations can be incorporated at numerous codons in each of the tandem repeats to minimize homologous recombination among the repeats. One example of MUC1 repeat sequences using wobbled codons to minimize homology while retaining repeated amino acid sequence is set forth below in Table A.

TABLE A

R1	GGC	TCC	ACC	GCC	CCC	CCA	GCC	CAC	GGT	GTC	ACC	TCG	GCC	CCG	GAC	ACC	AGG	CCG	GCC	CCG	(SEQ.ID:2)
R2	GGC	AGT	ACT	GCA	CCA	CCG	GCA	CAT	GGC	GTA	ACA	TCA	GCA	CCT	GAT	ACA	AGA	CCT	GCA	CCT	(SEQ.ID:4)
R3	GGA	TCC	ACC	GCG	CCG	CCT	GCG	CAC	GGA	GTG	ACG	TCG	GCG	CCC	GAC	ACG	CGC	CCC	GCT	CCC	(SEQ.ID:5)
R4	GGG	TCA	ACA	GCT	CCT	CCC	GCT	CAT	GGG	GTT	ACT	TCT	GCT	CCA	GAT	ACT	CGC	CCA	GCT	CCA	(SEQ.ID:6)
R5	GGT	TCG	ACG	GCC	CCC	CCT	GCT	CAC	GGT	GTA	ACA	TCC	GCC	CCG	GAT	ACC	AGA	CCG	GCC	CCT	(SEQ.ID:7)
R6	GGC	AGC	ACC	GCA	CCG	CCC	GCA	CAC	GGG	GTC	ACA	AGC	GCG	CCA	GAC	ACT	CGA	CCT	GCG	CCA	(SEQ.ID:8)
R7	GGA	AGT	ACC	GCT	CCA	CCT	GCA	CAC	GGG	GTC	ACA	AGC	GCG	CCA	GAC	ACT	CGA	CCT	GCG	CCA	(SEQ.ID:9)
R8	GGG	TCG	ACT	GCC	CCT	CCG	GCG	CAT	GGT	GTG	ACC	TCA	GCT	CCT	GAC	ACA	AGG	CCA	GCC	CCA	(SEQ.ID:10)
R9	GGT	TCA	ACG	GCA	CCT	CCA	GCA	CAC	GGA	GTC	ACG	TCT	GCA	CCC	GAC	ACC	CGT	CCA	GCT	CCG	(SEQ.ID:11)
R10	GGT	AGT	ACA	GCG	CCA	CCC	GCA	CAT	GGC	GTC	ACG	AGC	GCT	CCG	GAT	ACG	AGA	CCG	GCG	CCT	(SEQ.ID:12)
	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	(SEQ.ID:1)

One can use the various sequences in any combination. Further, one does not need to use all 10 repeats.

Nucleotide homology can also be reduced by introducing changes to the amino acid sequence, preferably conservative amino acid substitutions into some of the tandem repeats. Immunogenic epitopes such as (SEQ ID NO:3) PDTRPAP would preferably be left intact, but valine codon 10 could be changed to leucine codons CTT, CTC, CTA, and CTG in different repeats.

An immunogenic MUC1 fragment according to the invention can be made by a variety of conventional methods. For example, the fragment can be made by cloning a desired portion of the full-length human MUC1 DNA sequence (see e.g., Merlo, et al., *supra*; and Abe, M., et al., *supra*). Restriction enzymes can be used to cleave the desired fragment. The immunogenic MUC1 DNA fragment can also be prepared by amplification by the Polymerase Chain Reaction (i.e., PCR). Use of cloning and PCR amplification techniques to make an immunogenic MUC1 fragment is disclosed in Example 1 which follows.

An immunogenic mini-MUC1 fragment in accordance with the present invention can be inserted into a suitable pox virus to produce a recombinant pox virus which encodes the intact fragment and is reasonably stable. Expression of the immunogenic MUC1 fragment can be readily determined by several methods, including assaying samples of a suitable target cell or tissue by SDS-PAGE gel electrophoresis followed by Coomassie blue or silver staining; Western blot using DF3 antibody, or other suitable immunological technique such as ELISA.

Live recombinant viruses expressing an immunogenic cell encoded tumor associated antigen can be used to induce an immune response against tumor cells which express the protein. These recombinant viruses may be administered by scarification, as was conventionally done for small pox vaccination, or by other routes appropriate to the recombinant virus used. These may include among others, intramuscular, intradermal, subcutaneous, and intravenous routes. Vaccination of a host organism with live recombinant vaccinia virus is followed by replication of the virus within the host.

For parenteral administration, the recombinant vectors will typically be injected in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline. Kits containing the vector and the means for injection can be used. The kit preferably contains instructions describing how to use the vector. In one embodiment, the kit contains a vector modified to include an immunomodulator or a separate vector containing the immunomodulator as described below. In addition the kit can contain an adjuvant.

10 A specific immune response to a tumor associated antigen can be generated by administering between about 10^5 - 10^9 pfu of the recombinant pox virus, constructed as discussed above to a host; more preferably one uses $\geq 10^7$ pfu. The preferred host is a human. At least one interval thereafter, which is preferably one to three months later, the immune response is boosted by administering additional antigen to the host. More preferably there is at least a second "boost" preferably at least one to three months after the first boost, more preferably 6-12 months after the first boost. The boosting antigen may be administered using the same pox virus vector, or as a whole protein, an immunogenic peptide fraction of the protein, another recombinant viral vector, or DNA encoding the protein or peptide. Preferably, different pox viral vectors are used. For example, vaccinia may be followed by an avipox such as fowlpox, or vice versa. Cytokines, e.g., IL-2, IL-6, IL-12, IL-15, or co-stimulatory molecules, e.g., B7.1, B7.2, may be used as biologic adjuvants. The cytokines can be administered systemically to the host. Either cytokines or co-stimulatory molecules can be co-administered via co-insertion of the genes encoding the molecules into the recombinant pox vector or a second recombinant poxvirus which is admixed with the recombinant poxvirus expressing the TAA.

30 Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 (Aquila), incomplete Freund's adjuvant or many others.

Alternatively, it will sometimes be useful to use a recombinant pox virus encoding the immunogenic MUC1 fragment which has been modified to include an immunomodulator, for example, DNA encoding a T-cell co-

stimulatory factor and/or a cytokine such as interleukin (IL) (e.g., IL-2, IL-4, IL-10, IL-12), an interferon (IFN) (e.g., IFN- γ), granulocyte macrophage colony stimulating factor (GM-CSF) or an accessory molecule (e.g. ICAM-1). The construction of such multivalent vectors such as pox viral vectors is within the level of skill in the art based upon the present disclosure. In some cases, co-expression of the immunomodulatory agent such as the T-cell co-stimulatory factor and the immunogenic fragment of MUC1 by multiple vectors may be desirable. It may be desirable to administer a substantially pure preparation of, e.g., the immunomodulator to boost vaccine efficacy.

In preferred embodiments after initial administrations of the viral vector by one pox a different pox virus, preferably from a different pox family will be used for the following administrations (i.e. boosts). For example, initial administrations by vaccinia or avipox would preferably be followed by boosts from an avipox or vaccina, respectively, or by a suipox.

Although initially generally less preferred in most cases, it may be desirable to use another DNA or RNA virus or vector to insert an immunogenic MUC1 DNA fragment into a subject host. Such an approach may be useful where multiple boosts are used and the subject is at risk of developing an antigenic reaction to the host pox vector. Exemplary of such vectors are DNA or RNA viruses such as retroviruses, adenoviruses, herpes viruses or DNA-based vectors (see generally, Cepko et al., *Cell* 37:1053-1062 (1984); Morin et al., *Proc. Natl. Acad. Sci. USA* 84:4626-4630 (1987); Lowe et al., *Proc. Natl. Acad. Sci. USA*, 84:3896-3900 (1987); Panicali & Paoletti, *Proc. Natl. Acad. Sci. USA*, 79:4927-4931(1982); Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415-7419 (1982)). In an alternative embodiment, one would prime first with a non-pox viral vector expressing mini-MUC1, or a DNA segment encoding mini-MUC1, followed by boosting, wherein at least one boost involves the use of pox vectors.

Further contemplated uses of the recombinant pox viruses disclosed herein include use in the production of antibodies, particularly monoclonal antibodies that are capable of specifically binding the immunogenic MUC1 fragments. More specifically, it can be desirable to produce the antibodies, e.g., to detect mucin glycosylation in tumor and pre-tumorous cells *in vitro*

and *in vivo*. The antibodies may be prepared by a variety of standard methods well-known to those skilled in the art. For example, cells expressing an immunogenic MUC1 fragment can be administered to an animal to induce production of polyclonal antibodies. Alternatively, 5 monoclonal antibodies which specifically bind an immunogenic MUC1 fragment can be prepared using hybridoma technology (see, e.g., Kohler et al., *Nature* 256: 495 (1975); Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY (1981)).

Cytotoxic T-cells specific for an immunogenic MUC1 fragment can be 10 established from peripheral blood mononuclear cells (PBMC) obtained from a host immunized as discussed above. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968). Washed PBMC are resuspended in a 15 complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Deer, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 µg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2×10^5 cells in complete medium in a volume of, for example, 100 µl are added into each well of a 20 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). The immunogenic MUC1 fragment can be added to the cultures in a final concentration of about 50 µg/ml and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 5 days. After removal of the media containing the fragment, the cultures are provided with fresh human IL-2 25 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the immunogenic MUC1 fragment (50 µg/ml) on day 16. 5×10^5 irradiated (4,000 rad) autologous PBMC are added in a volume of about 50 µl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with 30 human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

The cytotoxic T-cells can be cultured in accordance with known methods and then injected back into the host by a variety of means. Generally, between 1×10^5 and 2×10^{11} cytotoxic T-cells per infusion are

administered in, for example, one to three infusions of 200 to 250 ml each over a period of 30 to 60 minutes. After the completion of the infusions, the patient may be treated with recombinant interleukin-2 with a dose of 720,000 IU per kilogram of body weight intravenously every eight hours; some doses can be omitted depending on the patient's tolerance for the drug. In addition, after infusion, additional recombinant pox virus or immunogenic MUC1 fragment containing T-cell eliciting epitope(s) may be administered to the patient to further expand the T-cell number. The antigen or epitope may be formulated with an adjuvant and/or may be in a liposomal formulation.

The cytotoxic T-cells can also be modified by introduction of a viral vector containing a DNA encoding TNF and reintroduced into a host in an effort to enhance the anti-tumor activity of the cells.

REFERENCE EXAMPLE 1

Pox Viruses

A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins. Representative vaccinia virus strains such as Wyeth and MVA have been disclosed previously. (Cepko et al., *Cell* 37:1053-1062 (1984); Morin et al., *Proc. Natl. Acad. Sci. USA* 84:4626-4630 (1987); Lowe et al., *Proc. Natl. Acad. Sci. USA*, 84:3896-3900 (1987); Panicali & Paoletti, *Proc. Natl. Acad. Sci. USA*, 79:4927-4931(1982); Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415-7419 (1982)). Representative fowlpox and swinepox virus are available through the ATCC under accession numbers VR-229 and VR-363, respectively. The Wyeth strain of vaccinia is available through the ATCC under accession number VR-325.

DNA Vectors For *In Vivo* Recombination With A Parent Virus

Genes that code for desired carcinoma associated antigens are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for *in vivo* recombination with a pox virus.

In general, the DNA donor vector contains the following elements:

- (i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;
- (ii) a gene encoding a marker which allows selection of
5 prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);
- (iii) at least one gene encoding a desired protein located adjacent to a transcriptional promoter capable of directing the expression of the gene; and
- 10 (iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in W091/19803.

- 15 In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA
20 fragments. The donor plasmids can be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

- The donor vector preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be
25 used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al., *J. Virol.*, 62:1046 (1988); Falkner and Moss., *J. Virol.*, 62:1849 (1988); Franke et al., *Mol. Cell. Biol.*, 5:1918 (1985), as well as genes such as the *E. coli lacZ* gene, that permit identification of recombinant
30 viral plaques by colorimetric assay (Panicali et al., *Gene*, 47:193-199 (1986)).

Integration Of Foreign DNA Sequences Into The Viral Genome And Isolation Of Recombinants

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo dermal (CED) cells, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, W089/03429). Alternatively, the donor DNA can be directly ligated into the parental virus genome at a unique restriction site (Scheifflinger, et al. (1992) *Proc. Natl. Acad. Sci. (USA)* 89:9977-9981).

Following *in vivo* recombination or ligation, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing integrated DNA will be TK⁺ and can be selected on this basis (Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415 (1982)). Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing β -galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al., *Gene*, 47:193 (1986)).

Characterizing The Viral Antigens Expressed By Recombinant Viruses

Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), enzyme immunoassay (EIA), or functional assay such as CTL assay.

EXAMPLE 1

Construction of Recombinant Vaccinia Virus Encoding MUC1 Gene Sequences

A. Mini-MUC1 gene vector

5 The human DF3/MUC1 cDNA was constructed from two cloned cDNA segments [Merlo, et al., *supra*; Abe, M. et al., *surpa*]. A 1.8 kb EcoRI fragment of MUC1 cDNA reported as containing 10 tandem repeats and its 3' unique sequence was inserted into Bluescript™ plasmid (Stratagene, La Jolla, CA) at the EcoRI site and designated pBs-MUC1. The 5' end of the

10 MUC1 gene was generated from another MUC1 clone by PCR using MUC1 specific primers. The 200 base pair amplification fragment was inserted into pBs-MUC1 at the HindII and HindIII sites creating pBS-miMUC1 containing the "mini" MUC1 gene (sometimes referred to herein as "miMUC1").

15 However, DNA sequence analysis of the miMUC1 gene confirmed that this gene contained the appropriate signal and start site, but not 10 tandem repeats. Instead it contained 7 repeats that showed some variation. The DNA sequence of the repeated portion of the miMUC1 gene is set forth below in Table B.

The deduced amino acid sequence of the repeat region predicted from

20 nucleotide sequence analysis of MUC1 gene is set forth below in Table C.

Moreover, the 3' coding sequence actually differs from that reported by Merlo, *supra*, but conforms to the 3' sequence reported by Gendler, *supra*. The entire coding sequence of the miniMUC1 gene is shown in Table D.

TABLE B

R1	GGC TCC ACC GCA CCC CCA GGC TCC ACC GGT GTC ACC TCG GCC CCG GAC ACC AGG CCG GCC CCG	(SEQ.ID NO:2)
R2	GGC TCC ACC GCC CCC CCA GCC CAC GGT GTC ACC TCG GCC CCG GAC ACC AGG CCG GCC CCG	(SEQ.ID NO:2)
R3	GGC TCC ACC GCC CCC CCA GCC CAC GGT GTC ACC TCG GCC CCG GAC ACC AGG CCG GCC CCG	(SEQ.ID NO:2)
R4	GGC TCC ACC GCC CCC CCA GCC CAC GGT GTC ACC TCG GCC CCG GAC ACC AGG CCG GCC CCG	(SEQ.ID NO:2)
R5	GGC TCC ACC GCA CCC CCA GGC TCC ACC GGT GTC ACC TCG GCC CCG GAC ACC AGG CCG GCC CCG	(SEQ.ID NO:13)
R6	GGC TCC ACC GCC CCC CCA GCC CAT GGT GTC ACC TCG GCC CCG GAC AAC AGG CCC GCC TTG	(SEQ.ID NO:14)
R7	GGC TCC ACC GCC CCT CCA GTC CAC AAT GTC ACC TCG GCC	(SEQ.ID NO:15)

TABLE C

Repeat 1	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	(SEQ.ID NO:1)
Repeat 2	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	(SEQ.ID NO:1)
Repeat 3	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	(SEQ.ID NO:1)
Repeat 4	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	R	A	P	(SEQ.ID NO:16)
Repeat 5:	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	(SEQ.ID NO:21)
Repeat 6:	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	N	R	P	A	L	(SEQ.ID NO:17)
Repeat 7:	G	S	T	A	P	P	A	H	N	V	T	S	A	P	D	N	R	P	A	L	(SEQ.ID NO:18)

TABLE D

ATGACACCGGGCACCCAGTCTCCTTCTCCTGCTGCTGCTCCTCAGTGCTT	(SEQ.ID:19)
M T P G T Q S P F L L L L T V L	
ACAGCTACCACAGCCCCCTAAACCCGCAACAGTTGTTACGGTTCTGGTCATGCA	(SEQ.ID:20)
T A T T A P K P A T V V T G S G H A	
AGCTCTACCCAGGTGGAGAAAGGAGACTTCGGCTACCCAGAGAA GTTCAGTG	(SEQ.ID:21)
S S T P G G E K E T S A T Q R S S V	
CCCAGCTCTACTGAGAAGAATGCTGTGAGTATGACAAGCTTGATATCGAATTCC	(SEQ.ID:22)
P S S T E K N A V S M T S L I S N S	
GGTGTCGGGGCTCCACCGCCCCCCCCAGCCACGGTGTCACTCGGCCCCCGGAC	(SEQ.ID:23)
G V R G S T A P P A H G V T S A P D	
ACCAGGCCGGCCCCGGGCTCCACCGCCCCCCCCAGCCACGGTGTCACTCGGCC	(SEQ.ID:24)
T R P A P G S T A P P A H G V T S A	
CCGACACACAGGCCGGCCCCGGGCTCCACCGCCCCCCCCAGCCACGGTGTCACT	(SEQ.ID:25)
P D T R P A P G S T A P P A H G V T	
TCGGCCCCGGACACACAGCGCGGCCCGGGCTCCACCGCACCCCCAGCCACCGGT	(SEQ.ID:26)
S A P D T R P A P G S T A P P A H G	
GTCACCTCGGCCCGGACACACAGCGCGGCCCGGGCTCCACCGCCCCCGGGC	(SEQ.ID:27)
V T S A P D T R R A P G S T P A P G	
TCCACCGCCCCCAGCCACGGTGTCACTCGGCCCGGACACACAGCGCCGCC	(SEQ.ID:28)
S T A P P A H G V T S A P D T R P A	
CCGGGCTCCACCGCCCCCAGCCCATGTGTCACTCGGCCCGGACACACAGG	(SEQ.ID:29)
P G S T A P P A H G V T S A P D N R	
CCCGCCTTGGGCTCCACCGCCCCCTCCAGTCCACAATGTCACTCGGCCCTCAGGC	(SEQ.ID:30)
P A L G S T A P P V H N V T S A S G	
TCTGCATCAGGCTCAGCTTCTACTCTGTGTGCACAACGGCACCTCTGCCAGGGCT	(SEQ.ID:31)
S A S G S A S T L V H N G T S A R A	
ACCACAACCCAGCCAGCAAGAGCACTCCATTCTCAATTCCCAGCCACCACTCT	(SEQ.ID:32)
T T T P A S K S T P F S I P S H S	
GATACTCCTACCACTTGCCAGCCATAGCACCAAGACTGATGCCAGTAGCACT	(SEQ.ID:33)
D' T P T T L A S H S T K T D A S S T	

TABLE D (cont'd)

CACCATAGCAGGTACCTCCTCTCACCTCCTCCAATCACAGCACCTTCTCCCCAG	(SEQ.ID:34)
H H S T V P P L T S S N H S T S P Q	(SEQ.ID:35)
TTGTCTACTGGGCTCTCTTTCTTTTCCCTGTCTTTTCACATTTCAAACCTCCAG	(SEQ.ID:36)
L S T G V S F F L S F H I S N L Q	(SEQ.ID:37)
TTTCCTTCCTCTCTCGAAGATCCCAGCACCGACTACTACCAAGAGCTGCAGAGA	(SEQ.ID:38)
F P S S L E D P S T D Y Y Q E L Q R	(SEQ.ID:39)
GACATTTCTCAAATGTTTTCGAGATTATATAACAAGGGGTTTCTGGGCCCTC	(SEQ.ID:40)
D I S Q M F L Q I Y K Q G G F L G L	(SEQ.ID:41)
TCCAATATTAAAGTTCAGGCCAGGATCTGTGTGGTACAATTGACTCTGGCCCTTC	(SEQ.ID:42)
S N I K F R P G S V L V Q L T L A F	(SEQ.ID:43)
CGAGAAGGTACCATCAATGTCCACGACGTCGGAGACACAGTTCATCAGTATAAA	(SEQ.ID:44)
R E G T I N V H D V E T Q F N Q Y K	(SEQ.ID:45)
ACGGAAGCAGCCTCTCGATATAACCTGACGATCCCAGACGTCAGCGTGAGTGAT	(SEQ.ID:46)
T E A A S R Y N L T I P D V S V S D	(SEQ.ID:47)
GTGCCATTTCTCTCTGCCCCAGTCTGGGGCTGGGGTGCCAGGCTGGGGGCATC	
V P F P F S A Q S G A G V P G W G I	
GCGCTGCTCCTGCTGTGTGTGTGTGTGTGCGCTGGCCATTGTCTATCTCATTT	
A L L L L V C V L V A L A I V Y L I	
GCCTTGGCTGTCTGTCTAGTGCCGCCGCGAAAGAACTACGGGCAGCTGGACATCTTT	
A L A V C Q C R R K N Y G Q L D I F	
CCAGCCCGGATACCTACCATCCTATGAGCGAGTACCCACCTACCACACCCCAT	
P A R D T Y H P M S E Y P T Y H T H	
GGGCGCTATGTCCCCCTAGCAGTACCGATCGTAGCCCCCTATGAGAAGGTTTCT	
G R Y V P P S S T D R S P Y E K V S	
GCAGGTAATGGTGGCAGCAGCCTCTCTTACACAAACCCAGCAGTGGCAGCCACT	
A G N G G S S L S Y T N P A V A A T	
TCTGCCAACTGTAG	
S A N L .	

B. Recombinant Vaccinia Virus

The miMUC1 gene described above was inserted 3' to the vaccinia 40K early/late promoter and flanked by sequences from the Hind III M region of the vaccinia genome. The resulting plasmid, designated pT2041, contained the miMUC1 gene under the control of the vaccinia virus 40K early/late promoter flanked by DNA sequences from the Hind III M region of the vaccinia genome. These flanking sequences included the vaccinia K1L host range gene required for growth of vaccinia virus on rabbit kidney RK13 cells (ATCC CCL37). A plaque-purified derivative of the Wyeth strain of vaccinia was used as the parental virus (designated vTBC33), lacked a functional K1L gene and thus could not efficiently replicate on RK13 cells. See e.g., Gritz, L., et al. (1990) *J. Virol.* 64:5948-57; Gillard, S., et al. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:5573-7; and Smith, KA., et al., *supra*.

Generation of recombinant vaccinia virus was accomplished via homologous recombination between vaccinia sequences in the vTBC33 genome and the corresponding sequences in pT2041 in vaccinia- infected RK13 cells transfected with pT2041. Recombinant virus, designated vT46(rV-MUC1), was selected by growth on RK13 cells (ATCC CCL37). Virus stocks were prepared by clarifying infected RK13 cell lysates followed by centrifugation through a 36% sucrose cushion.

The selection and screening of rV-MUC1 was done by growth in RK13 cells. The recombinant vaccinia rV-MUC1 was isolated as a single recombinant clone and purified by two rounds of plaque purification. The miMUC1 gene insertion into the vaccinia virus genome HindIIIM site by homologous recombination was confirmed by Southern analysis with ³²p radiolabeled miMUC1 gene as a probe. The Southern analysis indicated that the vaccinia virus had not deleted any portions of the gene, in contrast to the deletions reported with full-length MUC1 genes (see Bu, D., et al. *supra*).

A plasmid similar to pT2041 was constructed that contained the *lacZ* gene in addition to the mini-MUC1 gene; this plasmid was designated pT2068. The plasmid DNA pT2068 was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, under the conditions of the "Budapest Treaty on the International

Recognition of the Report of Microorganisms for the Purpose of Patent Procedure." The deposit was given ATCC Designation 97893. If the culture dies or is destroyed during the effective term, it will be replaced if a patent issues. If a patent issues, the strain will be maintained for 30 years from
5 the date of deposit, or five years after the most recent request, whichever is longer.

PT2068 was used to construct a recombinant vaccinia virus containing mini-MUC1 using a colorimetric screen for β -gal.

A recombinant vaccinia virus strain expressing the human co-
10 stimulatory molecule B7-1 (designated rV-B7) has been disclosed. The virus was grown in spinner cultures of HeLa cells, directly pelleted by centrifugation, and purified over 20%-40% sucrose gradients (Hodge, J.W., et al. (1994) *Cancer Res.* 54:5552-5; Earl, P.L., et al. (1993) *Generation of recombinant vaccinia viruses*, Vol.2, Suppl. 21, 16.17.1-16.18.10. New York:
15 John Wiley & Sons).

EXAMPLE 2

Characterization of Recombinant Vaccinia Virus With miMUC1 Insert

A. Southern Blot Hybridization Analysis

20 BSC-1 cells (ATCC CC126) were infected at an MOI of 10 with either miMUC1 recombinant vaccinia virus (designated rV-MUC1) or V-Wyeth. The viral DNA extraction, restriction endonuclease digestion and Southern blotting was performed as previously described (see e.g., Kaufman, H., et al. (1991) *Int. J. Cancer.* 48:900-7). The results indicated that the miMUC1
25 gene was stably inserted into the HindIIIIM region of the vaccinia genome.

B. Western Analysis of Protein Expression and Stability

Parallel confluent BSC- 1 cells were infected with either parental wild type vaccinia virus (V-Wyeth), or rV-MUC1 at an MOI of 5 in Dulbecco's
30 modified Eagle's medium containing 2.5 %FBS. After an overnight infection, cells in one plate were scraped and lysed in hypotonic lysis buffer (100 mM Tris-HCl pH 8.0, 100mM NaCl, 0.5% NP-40, and 0.2 mM PMSF). The infected cells in the corresponding parallel plate were scraped and high titer virus preparations were derived as previously described (Earl, P.L., et

al. (1993) *Generation of recombinant vaccinia viruses*, Vol.2, Supplement 21, 16.17.1-16.18.10. New York: John Wiley & Sons). The resulting virus was used to infect parallel confluent BSC- 1 cells as before. This cycle was repeated to generate protein extracts from cells infected with rV-MUC1 that
5 had gone through 2, 3, 4, and 5 passages of viral replication. Cell lysates were electrophoresed on an SDS-10% acrylamide gel. Proteins were electroblotted onto nitrocellulose, blocked, incubated with DF3 antibody (Kufe, D., et al. *supra*) for 4 hours at room temperature, washed and incubated with goat anti-mouse phosphatase labeled secondary antibody
10 (Kirkegaard and Perry, Gaithersburg, MD) and developed according to the manufacturer's instructions.

Stable expression of MUC1 after 2, 3, 4 or 5 passages of viral replication of rV-MUC1 was confirmed by Western analysis (Fig. 1). Incubation of protein extracted from rV-MUC1 infected cells from passage 2
15 (Fig. 1, lane 2) with the monoclonal antibody DF3 revealed a broad 150-175 kD band. Similarly, incubation of protein extracted from cells infected with viral passages 3, 4, and 5 with DF3 (lanes 3, 4, and 5) revealed identical bands ranging from 150-175 kD. Lane 1 contains purified MUC1 protein of approximately 300kD. This finding is consistent with reports indicating the
20 apparent molecular mass of these glycoproteins, which appear heterogeneous as a result of O-linked glycosylation in the tandem repeats (Sekine, H., et al. (1985) *J. Immunol.* 135:3610-5). Uninfected or V-Wyeth infected cells were negative for the expression of MUC1 by Western blot using DF3 MAb.

25

EXAMPLE 3

Construction and Characterization of Recombinant Vaccinia Virus Containing MUC1 and B7.1

The miMUC1 gene and the human B7.1 gene were each ligated to
30 vaccinia promoters. The promoter-gene cassettes were then inserted into a plasmid vector containing the *E. coli lacZ* gene flanked by DNA sequences from the HindIII M region of the vaccinia genome. The resulting plasmid, designated pT2043, contains the B7.1 gene under the control of the vaccinia virus 30K promoter (located at the HindIII M insertion site; Perkus

et al. (1985) *Science* 229: 981-984), the MUC1 gene under the control of the vaccinia virus 40K early/late promoter (Gritz *et al.*, *supra*), and the *lacZ* gene under the control of the fowlpox Cl promoter (Jenkins *et al.*, (1991) *AIDS Res. Human Retrovirus* 7:991-998), all flanked by DNA sequences

5 from the HindIII M region of the vaccinia genome. A plaque-purified derivative of the Wyeth strain of vaccinia was used as the parental virus in the construction of recombinant vaccinia virus. The generation of recombinant vaccinia virus was accomplished via homologous recombination between vaccinia sequences in the Wyeth vaccinia genome

10 and the corresponding sequences in pT2043 in vaccinia-infected RK₁₃ cells transfected with pT2043. Recombinant virus, designated vT2043, was identified using a chromogenic substrate for β -galactosidase (Bluo-GalTM). Viral plaques expressing *lacZ* appeared blue against a clear background. Positive plaques were picked from the cell monolayer and their progeny were

15 further propagated. Repeated rounds of plaque isolation and replating in the presence of Bluo-Gal resulted in the purification of the desired recombinant. Virus stocks were prepared by clarifying infected RK₁₃ cell lysates followed by centrifugation through a 36% sucrose cushion. Insertion of the MUC1 and B7.1 genes into the vaccinia genome was confirmed by

20 Southern analysis using MUC1 and B7.1 gene probes. Expression of MUC1 and B7.1 protein was demonstrated by Western analysis using antibodies specific for each protein. More preferably, another cell line such as the monkey kidney cell line CV-1 (ATCC CCL 70) or chick embryo dermal (CED) cells would be used for vaccine production.

25

EXAMPLE 4

Construction and Characterization of Recombinant Avipox Virus Containing MUC1 and B7.1

The miMUC1 gene is inserted into a plasmid vector containing the

30 vaccinia 40K promoter and the *E. coli lacZ* gene flanked by DNA sequences from the BamHI J region of the fowlpox genome. The resulting plasmid contains the miMUC1 gene under the control of the vaccinia virus 40K early/late promoter (Gritz *et al.*, *supra*), and the *lacZ* gene under the control of the fowlpox Cl promoter (Jenkins *et al.*, *supra*), all flanked by DNA

sequences from the BamHI J region of the fowlpox genome. The parental virus used for the generation of this recombinant virus is the USDA licensed live fowlpox vaccine POXVAC-TC (Schering-Plough Corporation). The generation of recombinant vaccinia virus is accomplished via homologous recombination between fowlpox sequences in the POXVAC-TC fowlpox genome and the corresponding sequences in the plasmid vector in fowlpox-infected chick embryo dermal cells (CED), prepared as described (Jenkins *et al.*, supra), transfected with the plasmid vector. Recombinant virus is identified using a chromogenic substrate for β -galactosidase (Bluo-GalTM).
10 Viral plaques expressing *lacZ* appear blue against a clear background. Positive plaques are picked from the cell monolayer and their progeny are further propagated. Repeated rounds of plaque isolation and replating in the presence of Bluo-Gal result in the purification of the desired recombinant. Virus stocks are prepared by clarifying infected CED cell
15 lysates followed by centrifugation through a 20% sucrose cushion. Insertion of the MUC1 gene into the fowlpox genome is confirmed by Southern analysis using a MUC1 gene probe. Expression of MUC1 protein is demonstrated by Western analysis using antibodies specific for MUC1.

Construction and characterization of a recombinant fowlpox virus containing both MUC1 and B7.1 is accomplished by inserting a promoter-B7.1 cassette into the plasmid described above, and by carrying out the manipulations described above.

Recombinant canary pox viruses containing MUC1 or MUC1 and B7.1 are constructed and characterized in an analogous fashion using canary pox as the parental virus (Taylor *et al.* (1991) Vaccine 9:190-193; Paoletti, U.S. patent 5,505,941).

EXAMPLE 5

1. Transfection and Transduction of the miMUC1 gene in pLNSX
30 A 2 kb XhoI/XbaI restriction endonuclease fragment from pBs-miMUC1 was isolated and the ends repaired with DNA polymerase 1-Klenow fragment and ligated into the Stu I site of the retroviral vector pLNSX. The pLNSX-miMUC1 gene was transfected into the PA317 packaging cell line by Lipofectin (GIBCO/BRL) according to the

manufacturer's instructions. Cells were harvested, plated onto 60mm dishes, and incubated with 200-500 μ g/ml G418 for three weeks. Clones of PA317 cells containing the miMUC1 gene were identified by Northern blot analysis of total RNA isolated from G418 resistant clones using the

5 *Xba*I/*Xho*I DNA fragment of the miMUC1 gene as a radioactive probe. The retroviral supernatants of MUC1-transduced PA317 cells were collected and used to transduce MC38 cells in the presence of polybrene (8 μ g/ml). Following transduction, MC38 cells were selected by cloning G418 resistant colonies and selection by FACs analysis using DF3 antibody. The resultant

10 MUC1 positive cell line was designated MC38/MUC1. Those cells were shown to be negative for B7-1 expression by flow cytometry.

The amphotrophic packaging cell line PA317 was obtained from Dr. Robert Bassin (National Cancer Institute, NIH, Bethesda, MD).

The MC38 murine colonic adenocarcinoma cell line (20) was obtained

15 from the laboratory of Dr. Steve Rosenberg (National Cancer Institute, NIH, Bethesda, MD).

EXAMPLE 6

FACS Analysis of Recombinant Protein Expression

20 Cell surface expression of MUC1 on MC38/MUC1 cells was analyzed by immunofluorescence. Cells were harvested and incubated at 4°C for 30 minutes with 1 μ g/ml DF3 MAb in 5% FBS-DPBS, followed by incubation with fluorescein-labeled goat anti-mouse IgG (Kirkegaard and Perry) for 30 minutes at 4°C. Analysis was performed with a FACScan (Becton-Dickinson

25 Mountain View, CA).

Surface expression of MUC1 glycoprotein in MUC1-transduced MC38 cells was examined by flow cytometry. Figs. 2A and 2B illustrate that untransduced MC38 cells (Fig. 2A) do not react with DF3 MAb (98.5% of the cells are negative with a mean fluorescence of 20). However, MC38 cells

30 transduced with the MUC1 gene (Fig. 2B) react strongly with the DF3 antibody (87.5% of the cells are positive with a mean fluorescence of 400). These studies thus demonstrate that MC38 tumor cells transduced with the miMUC1 gene (MC38/MUC1) express the MUC1 molecule.

EXAMPLE 7

Anti-Tumor Activity of Recombinant Vaccinia Virus Vaccine

- A. Cytotoxicity Assay. To analyze the effect of rV-MUC1 or rV-MUC1/rV-B7 vaccination on MUC1 specific cytotoxic activity, splenic lymphocytes
- 5 from mice inoculated with rV-MUC1 or the mixture of rV-MUC1 and rV-B7 were tested for their ability to lyse murine adenocarcinoma cells that were negative (MC38) or positive for MUC1 (MC38/MUC1) (Kantor, 3., et al. *supra*). Briefly, spleens were removed and mechanically dispersed through
- 10 70 mm cell strainers (Falcon, Becton Dickinson, Franklin Lakes, NJ) to isolate single cell suspensions. Erythrocytes and dead cells were removed by centrifugation over a Ficoll-Hypaque gradient (density = 1.119 g/ml) (Sigma Chemical Co., St. Louis, MO). MC38 cells and MC38/MUC1 cells were prepared for use as targets in a standard indium release assay as described previously (Hodge, J.W., et al. (1995) *Cancer Res.* 55:3598-603).
- 15 Tumor cells (2×10^6 cells) were radiolabeled with 50 μ Ci of ^{111}In oxyquinoline solution (Amersham, Arlington Heights, IL) for 30 minutes at 37°C and dispensed (10^4 cells/50 μ l) into each well of 96-well U-bottom plates (Costar, Cambridge, MA). T-cells were added to effector to target (E:T) ratios of 100:1 - 12.5:1 in 96 well U-bottomed plates (Costar) and incubated
- 20 for 16 hours at 37°C with 5% CO_2 . After incubation, supernants were collected using a Supernatant Collection System (Skatron, Sterling, VA) and radioactivity was quantitated using a gamma counter. (Cobra Autogamma, Packard, Downers Grove, IL). The percentage of specific release of ^{111}In was determined by the standard equation: % specific lysis = [(experimental-spontaneous)/(maximum-spontaneous)] $\times 100$.
- 25

Fig. 3 shows the results of an experiment in which groups of mice were inoculated with an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU V-Wyeth (squares), or an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU rV-B7 (circles). All groups were inoculated with an admixture of 10^7 PFU rV-MUC1

30 and 10^7 PFU V-Wyeth after 14 and 28 days. Seven days following the final immunization, cytolytic activity was quantified against MC38 cells (MUC1 negative; closed symbols) or MC38/MUC1 cells (MUC1 positive; open symbols). T-cells from mice inoculated three times with rV-MUC1/V-Wyeth or one time with rV-MUC1/rV-B7 followed by two inoculations with rV-

MUC1/V-Wyeth did not lyse the MUC1 negative MC38 targets (closed symbols), but did lyse the MUC1 positive MC38/MUC1 targets (open symbols). This MUC1 specific lysis was E:T ratio dependent.

5 B. Prevention of MUC1 Positive Pulmonary Metastases

Groups of C57BL/6 mice were inoculated subcutaneously with either (a) an admixture of 10^7 PFU rV-B7 and 10^7 PFU V-Wyeth; (b) an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU V-Wyeth; or (c) an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU rV-B7. After two weeks, mice in the first group were
10 inoculated with 2×10^7 PFU V-Wyeth; while the remaining two groups were inoculated with an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU V-Wyeth. Two weeks later, mice were challenged intravenously with 2×10^6 MC38/MUC1 tumor cells. Mice were euthanized 28 days following tumor transplant and experimental pulmonary metastatic nodules as defined by
15 Wexler (Wexler, H., et al. (1966) *J. Natl. Cancer Inst.* 36:641-645) were stained. These metastatic nodules were enumerated in a blind fashion, and lungs with nodules too numerous to count were assigned an arbitrary value of >250.

Fig. 4 illustrates the efficacy of rV-MUC1 in this experimental tumor
20 model. Mice inoculated with rV-B7/V-Wyeth were all positive for lung metastases (8/10 mice had greater than 250 nodules). In contrast, 90% of mice inoculated with rV-MUC1/V-Wyeth and boosted with rV-MUC1/V-Wyeth remained free of pulmonary metastases (1/10 mice had 6 nodules). Similarly, 90% of mice receiving the same immunization scheme with the
25 addition of rV-B7 in the first immunization remained free of pulmonary metastases.

C. Therapy of Established MUC1 Positive Pulmonary Metastases

C57BL/6 mice were challenged intravenously with 2×10^6
30 MC38/MUC1 tumor cells. After 3 days, mice were randomized and inoculated subcutaneously with either (a) 2×10^7 PFU V-Wyeth; (b) an admixture of 10^7 PFU rV-B7 and 10^7 PFU V-Wyeth; (c) an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU V-Wyeth; or (d) an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU rV-B7. Seven days later, mice in the first two groups

were inoculated intravenously with 2×10^7 PFU V-Wyeth, while the remaining two groups were inoculated intravenously with an admixture of 10^7 PFU rV-MUC1 and 10^7 PFU V-Wyeth. Seven days later, following this inoculation, mice were inoculated a third time in a similar fashion. Mice were euthanized 28 days following tumor transplant and pulmonary metastatic nodules were stained and enumerated as above. Identically treated groups were followed for survival. Kaplan-Meier plots and Mantel-Cos (Logrank) tests were used to compare survival of mice belonging to different treatment groups.

Fig. 5 shows the efficacy of rV-MUC1 in a therapeutic setting. Mice inoculated with V-Wyeth or rV-B7/V-Wyeth were all positive for lung metastases (9/10 and 7/10, respectively had greater than 250 nodules.) Although all mice inoculated 3 times with rV-MUC1/V-Wyeth were positive for lung nodules, the number of metastases was comparatively low (7/10 with <50 nodules). In contrast, 30% of mice inoculated with rV-MUC1/rV-B7 and boosted with rV-MUC1/V-Wyeth remained free of pulmonary metastases, while the remaining mice all had less than 20 lung nodules.

Fig. 6 depicts a different parameter of therapy of established MUC1 positive pulmonary metastases in which a parallel group of mice was inoculated identically to those above and monitored for survival. In the experiment shown in Fig. 6, groups of 10 mice were transplanted intravenously with 2×10^6 MC38/MUC1 tumor cells, and tumors were allowed to establish for 3 days. Mice were inoculated every 7 days as in Fig. 5. Immunization sequences were: V-Wyeth: V-Wyeth: V-Wyeth (open triangles); rV-B7/V-Wyeth: V-Wyeth: V-Wyeth (closed circles); rV-MUC1/V-Wyeth: rV-MUC1/V-Wyeth: rV-MUC1/V-Wyeth: rV-MUC1/V-Wyeth: rV-MUC1/V-Wyeth (closed squares); and rV-MUC1/rV-B7: rV-MUC1/V-Wyeth: rV-MUC1/V-Wyeth (open circles). Vaccination of mice with V-Wyeth or rV-B7/V-Wyeth and boosting with V-Wyeth had no effect on mouse survival, with 100% mortality by 50-56 days post tumor challenge. In contrast, inoculation of mice three times with rV-MUC1/V-Wyeth resulted in a significant improvement of survival time ($p=0.0009$ - $<.0001$). Furthermore, immunization of mice with rV-MUC1/rV-B7 followed by two boosts with rV-MUC1/V-Wyeth resulted in 100% survival of mice ($p<.0001$). It thus

appears that the administration of rV-MUC1 can significantly improve the survival of mice bearing MUC1 positive tumors, but only the admixture of rV-MUC1 with rV-B7 can completely eradicate MUC1 expressing tumors. Lungs of these animals were examined at day 65 and were free of tumor
5 nodules.

The recombinant pox viruses of the present invention provide significant advantages. For example, previously described recombinant vaccinia viruses encoding MUC1 can undergo significant genetic deletion thereby destabilizing the virus, decreasing antigen immunogenicity, and
10 reducing vaccine efficacy. In contrast, the present recombinant pox viruses encode an immunogenic MUC1 fragment that does not undergo significant genetic deletion, thereby providing an unexpectedly stable and immunogenic pox virus. Accordingly, efficacy of vaccines including the present recombinant pox viruses is substantially increased. Propagation of
15 the present recombinant pox viruses is positively impacted by the stability of the immunogenic MUC1 fragment, e.g., by providing uniform isolation of desired viral strains. Importantly, vaccine efficacy against established or pre-existing MUC1 expressing tumors is potentiated by providing an immunomodulator such as a T-cell co-stimulatory factor, particularly as an
20 admixture with another recombinant pox virus encoding the T-cell co-stimulatory factor.

All publications, patents, and patent applications mentioned in the specification are indicative of the level of skill of those in the art to which
25 this invention pertains.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

32a

SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANT: Therion Biologics Corporation et al.
- (ii) TITLE OF THE INVENTION: Recombinant Pox Virus For
Immunization Against MUC1 Tumor-Associated Antigen
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Swabey Ogilvy Renault
 - (B) STREET: 1981 McGill College, suite 1600
 - (C) CITY: Montreal
 - (D) STATE: Quebec
 - (E) COUNTRY: Canada
 - (F) ZIP: H3A 2Y3
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 24-FEB-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US98/03693
 - (B) FILING DATE: 24-FEB-1998
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/038,253
 - (B) FILING DATE: 24-FEB-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: CÔTÉ, France
 - (B) REGISTRATION NUMBER: 4166
 - (C) REFERENCE/DOCKET NUMBER: 13297-28 FC/ntb
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (514) 845-7126
 - (B) TELEFAX: (514) 288-8389
 - (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid

32b

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr
1				5				10						15	
Arg	Pro	Ala	Pro												
			20												

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGCTCCACCG CCCCCCAGC CCACGGTGTC ACCTCGGCCC CGGACACCAG GCCGGCCCCG 60

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro	Asp	Thr	Arg	Pro	Ala	Pro
1				5		

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCAGTACTG CACCACCGGC ACATGGCGTA ACATCAGCAC CTGATACAAG ACCTGCACCT 60

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

32c

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGATCCACCG CGCCGCCTGC GCACGGAGTG ACGTCGGCGC CCGACACGCG CCCCCTCCC 60

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGTCAACAG CTCCTCCCGC TCATGGGGTT ACTTCTGCTC CAGATACTCG CCCAGCTCCA 60

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGTTCGACGG CCCCCCTGC TCACGGTGTA ACATCCGCCC CGGATACCAG ACCGGCCCCT 60

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCAGCACCG CACCGCCCGC ACACGGGGTC ACAAGCGCGC CAGACACTCG ACCTGCGCCA 60

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid

32d

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGAAGTACCG CTCCACCTGC ACACGGGGTC ACAAGCGCGC CAGACACTCG ACCTGCGCCA 60

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGTCGACTG CCCCTCCGGC GCATGGTGTG ACCTCAGCTC CTGACACAAG GCCAGCCCCA 60

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGTTCAACGG CACCTCCAGC ACACGGAGTC ACGTCTGCAC CCGACACCCG TCCAGCTCCG 60

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTAGTACAG CGCCACCCGC ACATGGCGTC ACGAGCGCTC CGGATACGAG ACCGGCGCCT 60

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

32e

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCCACCG CACCCCCAGC CCACGGTGTC ACCTCGGCCC CGGACACCAG GCGGGCCCCG 60
 GGCTCCACCC CGGCCCCG 78

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCTCCACCG CCCCCCAGC CCATGGTGTC ACCTCGGCCC CGGACAACAG GCCCGCCTTG 60

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCCACCG CCCCTCCAGT CCACAATGTC ACCTCGGCC 39

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr
1				5				10					15		
Arg	Arg	Ala	Pro												
			20												

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids

32f

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Asn
1				5				10						15	
Arg	Pro	Ala	Leu												
			20												

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gly	Ser	Thr	Ala	Pro	Pro	Val	His	Asn	Val	Thr	Ser	Ala
1				5				10				

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1527 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 1...1524
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATG	ACA	CCG	GGC	ACC	CAG	TCT	CCT	TTC	TTC	CTG	CTG	CTG	CTC	CTC	ACA	48
Met	Thr	Pro	Gly	Thr	Gln	Ser	Pro	Phe	Phe	Leu	Leu	Leu	Leu	Leu	Thr	
1				5				10							15	
GTG	CTT	ACA	GCT	ACC	ACA	GCC	CCT	AAA	CCC	GCA	ACA	GTT	GTT	ACG	GGT	96
Val	Leu	Thr	Ala	Thr	Thr	Ala	Pro	Lys	Pro	Ala	Thr	Val	Val	Thr	Gly	
			20				25						30			
TCT	GGT	CAT	GCA	AGC	TCT	ACC	CCA	GGT	GGA	GAA	AAG	GAG	ACT	TCG	GCT	144
Ser	Gly	His	Ala	Ser	Ser	Thr	Pro	Gly	Gly	Glu	Lys	Glu	Thr	Ser	Ala	
			35				40					45				

32g

ACC	CAG	AGA	AGT	TCA	GTG	CCC	AGC	TCT	ACT	GAG	AAG	AAT	GCT	GTG	AGT	192
Thr	Gln	Arg	Ser	Ser	Val	Pro	Ser	Ser	Thr	Glu	Lys	Asn	Ala	Val	Ser	
	50					55					60					
ATG	ACA	AGC	TTG	ATA	TCG	AAT	TCC	GGT	GTC	CGG	GGC	TCC	ACC	GCC	CCC	240
Met	Thr	Ser	Leu	Ile	Ser	Asn	Ser	Gly	Val	Arg	Gly	Ser	Thr	Ala	Pro	
65					70					75					80	
CCA	GCC	CAC	GGT	GTC	ACC	TCG	GCC	CCG	GAC	ACC	AGG	CCG	GCC	CCG	GGC	288
Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly	
				85					90					95		
TCC	ACC	GCC	CCC	CCA	GCC	CAC	GGT	GTC	ACC	TCG	GCC	CCG	GAC	ACC	AGG	336
Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	
			100					105					110			
CCG	GCC	CCG	GGC	TCC	ACC	GCC	CCC	CCA	GCC	CAC	GGT	GTC	ACC	TCG	GCC	384
Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	
		115					120					125				
CCG	GAC	ACC	AGG	CCG	GCC	CCG	GGC	TCC	ACC	GCA	CCC	CCA	GCC	CAC	GGT	432
Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	
	130					135					140					
GTC	ACC	TCG	GCC	CCG	GAC	ACC	AGG	CGG	GCC	CCG	GGC	TCC	ACC	CCG	GCC	480
Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	Arg	Ala	Pro	Gly	Ser	Thr	Pro	Ala	
145					150					155					160	
CCG	GGC	TCC	ACC	GCC	CCC	CCA	GCC	CAC	GGT	GTC	ACC	TCG	GCC	CCG	GAC	528
Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	
				165					170					175		
ACC	AGG	CCG	GCC	CCG	GGC	TCC	ACC	GCC	CCC	CCA	GCC	CAT	GGT	GTC	ACC	576
Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	
			180					185					190			
TCG	GCC	CCG	GAC	AAC	AGG	CCC	GCC	TTG	GGC	TCC	ACC	GCC	CCT	CCA	GTC	624
Ser	Ala	Pro	Asp	Asn	Arg	Pro	Ala	Leu	Gly	Ser	Thr	Ala	Pro	Pro	Val	
		195				200						205				
CAC	AAT	GTC	ACC	TCG	GCC	TCA	GGC	TCT	GCA	TCA	GGC	TCA	GCT	TCT	ACT	672
His	Asn	Val	Thr	Ser	Ala	Ser	Gly	Ser	Ala	Ser	Gly	Ser	Ala	Ser	Thr	
	210					215					220					
CTG	GTG	CAC	AAC	GGC	ACC	TCT	GCC	AGG	GCT	ACC	ACA	ACC	CCA	GCC	AGC	720
Leu	Val	His	Asn	Gly	Thr	Ser	Ala	Arg	Ala	Thr	Thr	Thr	Pro	Ala	Ser	
225					230					235					240	
AAG	AGC	ACT	CCA	TTC	TCA	ATT	CCC	AGC	CAC	CAC	TCT	GAT	ACT	CCT	ACC	768
Lys	Ser	Thr	Pro	Phe	Ser	Ile	Pro	Ser	His	His	Ser	Asp	Thr	Pro	Thr	
				245					250					255		

32h

ACC	CTT	GCC	AGC	CAT	AGC	ACC	AAG	ACT	GAT	GCC	AGT	AGC	ACT	CAC	CAT	816
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			260					265					270			
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	290					295					300					
CTC	CAG	TTT	CCT	TCC	TCT	CTC	GAA	GAT	CCC	AGC	ACC	GAC	TAC	TAC	CAA	960
Leu	Gln	Phe	Pro	Ser	Ser	Leu	Glu	Asp	Pro	Ser	Thr	Asp	Tyr	Tyr	Gln	
305					310				315						320	
GAG	CTG	CAG	AGA	GAC	ATT	TCT	CAA	ATG	TTT	TTG	CAG	ATT	TAT	AAA	CAA	1008
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				325				330						335		
GGG	GGT	TTT	CTG	GGC	CTC	TCC	AAT	ATT	AAG	TTC	AGG	CCA	GGA	TCT	GTG	1056
Gly	Gly	Phe	Leu	Gly	Leu	Ser	Asn	Ile	Lys	Phe	Arg	Pro	Gly	Ser	Val	
			340					345					350			
CTG	GTA	CAA	TTG	ACT	CTG	GCC	TTC	CGA	GAA	GGT	ACC	ATC	AAT	GTC	CAC	1104
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		355					360					365				
GAC	GTG	GAG	ACA	CAG	TTC	AAT	CAG	TAT	AAA	ACG	GAA	GCA	GCC	TCT	CGA	1152
Asp	Val	Glu	Thr	Gln	Phe	Asn	Gln	Tyr	Lys	Thr	Glu	Ala	Ala	Ser	Arg	
	370					375					380					
TAT	AAC	CTG	ACG	ATC	CCA	GAC	GTC	AGC	GTG	AGT	GAT	GTG	CCA	TTT	CCT	1200
Tyr	Asn	Leu	Thr	Ile	Pro	Asp	Val	Ser	Val	Ser	Asp	Val	Pro	Phe	Pro	
385					390				395						400	
TTC	TCT	GCC	CAG	TCT	GGG	GCT	GGG	GTG	CCA	GGC	TGG	GGC	ATC	GCG	CTG	1248
Phe	Ser	Ala	Gln	Ser	Gly	Ala	Gly	Val	Pro	Gly	Trp	Gly	Ile	Ala	Leu	
			405					410						415		
CTC	CTG	CTG	GTC	TGT	GTT	CTG	GTT	GCG	CTG	GCC	ATT	GTC	TAT	CTC	ATT	1296
Leu	Leu	Leu	Val	Cys	Val	Leu	Val	Ala	Leu	Ala	Ile	Val	Tyr	Leu	Ile	
			420					425					430			
GCC	TTG	GCT	GTC	TGT	CAG	TGC	CGC	CGA	AAG	AAC	TAC	GGG	CAG	CTG	GAC	1344
Ala	Leu	Ala	Val	Cys	Gln	Cys	Arg	Arg	Lys	Asn	Tyr	Gly	Gln	Leu	Asp	
		435					440					445				
ATC	TTT	CCA	GCC	CGG	GAT	ACC	TAC	CAT	CCT	ATG	AGC	GAG	TAC	CCC	ACC	1392
Ile	Phe	Pro	Ala	Arg	Asp	Thr	Tyr	His	Pro	Met	Ser	Glu	Tyr	Pro	Thr	
	450					455					460					

(2) INFORMATION FOR SEO ID NO:20:

(A) LENGTH: 508 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(v) FRAGMENT TYPE: internal
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Val	Leu	Thr	Ala	Thr	Thr	Ala	Pro	Lys	Pro	Ala	Thr	Val	Val	Thr	Gly
			20					25					30		
Ser	Gly	His	Ala	Ser	Ser	Thr	Pro	Gly	Gly	Glu	Lys	Glu	Thr	Ser	Ala
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Thr	Gln	Arg	Ser	Ser	Val	Pro	Ser	Ser	Thr	Glu	Lys	Asn	Ala	Val	Ser
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Met	Thr	Ser	Leu	Ile	Ser	Asn	Ser	Gly	Val	Arg	Gly	Ser	Thr	Ala	Pro
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Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly
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Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg
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Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala
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Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly
	130					135					140				
Val	Thr	Ser	Ala	Pro	Asp	Thr	Arg	Arg	Ala	Pro	Gly	Ser	Thr	Pro	Ala
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Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr	Ser	Ala	Pro	Asp
				165					170					175	
Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro	Pro	Ala	His	Gly	Val	Thr
			180					185					190		
Ser	Ala	Pro	Asp	Asn	Arg	Pro	Ala	Leu	Gly	Ser	Thr	Ala	Pro	Pro	Val
		195					200					205			
His	Asn	Val	Thr	Ser	Ala	Ser	Gly	Ser	Ala	Ser	Gly	Ser	Ala	Ser	Thr
	210					215					220				

32j

Leu	Val	His	Asn	Gly	Thr	Ser	Ala	Arg	Ala	Thr	Thr	Thr	Pro	Ala	Ser	225		230		235		240
Lys	Ser	Thr	Pro	Phe	Ser	Ile	Pro	Ser	His	His	Ser	Asp	Thr	Pro	Thr		245		250		255	
Thr	Leu	Ala	Ser	His	Ser	Thr	Lys	Thr	Asp	Ala	Ser	Ser	Thr	His	His		260		265		270	
Ser	Thr	Val	Pro	Pro	Leu	Thr	Ser	Ser	Asn	His	Ser	Thr	Ser	Pro	Gln		275		280		285	
Leu	Ser	Thr	Gly	Val	Ser	Phe	Phe	Phe	Leu	Ser	Phe	His	Ile	Ser	Asn	290		295		300		
Leu	Gln	Phe	Pro	Ser	Ser	Leu	Glu	Asp	Pro	Ser	Thr	Asp	Tyr	Tyr	Gln	305		310		315		320
Glu	Leu	Gln	Arg	Asp	Ile	Ser	Gln	Met	Phe	Leu	Gln	Ile	Tyr	Lys	Gln		325		330		335	
Gly	Gly	Phe	Leu	Gly	Leu	Ser	Asn	Ile	Lys	Phe	Arg	Pro	Gly	Ser	Val		340		345		350	
Leu	Val	Gln	Leu	Thr	Leu	Ala	Phe	Arg	Glu	Gly	Thr	Ile	Asn	Val	His		355		360		365	
Asp	Val	Glu	Thr	Gln	Phe	Asn	Gln	Tyr	Lys	Thr	Glu	Ala	Ala	Ser	Arg	370		375		380		
Tyr	Asn	Leu	Thr	Ile	Pro	Asp	Val	Ser	Val	Ser	Asp	Val	Pro	Phe	Pro	385		390		395		400
Phe	Ser	Ala	Gln	Ser	Gly	Ala	Gly	Val	Pro	Gly	Trp	Gly	Ile	Ala	Leu		405		410		415	
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Ala	Leu	Ala	Val	Cys	Gln	Cys	Arg	Arg	Lys	Asn	Tyr	Gly	Gln	Leu	Asp		435		440		445	
Ile	Phe	Pro	Ala	Arg	Asp	Thr	Tyr	His	Pro	Met	Ser	Glu	Tyr	Pro	Thr	450		455		460		
Tyr	His	Thr	His	Gly	Arg	Tyr	Val	Pro	Pro	Ser	Ser	Thr	Asp	Arg	Ser	465		470		475		480
Pro	Tyr	Glu	Lys	Val	Ser	Ala	Gly	Asn	Gly	Gly	Ser	Ser	Leu	Ser	Tyr		485		490		495	
Thr	Asn	Pro	Ala	Val	Ala	Ala	Thr	Ser	Ala	Asn	Leu						500		505			

CLAIMS:

1. A recombinant pox virus comprising a nucleic acid sequence encoding an immunogenic MUC1 fragment comprising 5 to 25 MUC1 tandem repeat units, the nucleic acid sequence comprising a first nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 that is SEQ ID NO: 2; and a second nucleotide sequence encoding 4 to 24 copies of the amino acid sequence of SEQ ID NO: 1 wherein the second nucleotide sequence comprises 4 to 24 copies of an altered nucleotide sequence of SEQ ID NO: 2 that is altered by changing wobbled nucleotides of the codons of SEQ ID NO: 2, wherein the altered nucleotide sequence is selected from SEQ ID NOS: 4 to 12.
2. The recombinant pox virus of claim 1, wherein the immunogenic MUC1 fragment comprises 6 to 14 tandem repeat units.
3. The recombinant pox virus of claim 2, wherein the immunogenic MUC1 fragment comprises 9 tandem repeat units.
4. The recombinant pox virus of claim 1, wherein the pox virus is selected from the group consisting of orthopox, suipox and avipox.
5. A pharmaceutical composition comprising a recombinant pox virus comprising a nucleic acid sequence encoding an immunogenic MUC1 fragment comprising 5 to 25 MUC1 tandem repeat units, the nucleic acid sequence comprising a first nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 that is SEQ ID NO: 2; a second nucleotide sequence encoding 4 to 24 copies of the amino acid sequence of SEQ ID NO: 1 wherein the second nucleotide sequence comprises 4 to 24 copies of an altered nucleotide sequence of SEQ ID NO: 2 which is altered by changing wobbled nucleotides of the codons of SEQ ID NO: 2, wherein the altered nucleotide sequence is selected from SEQ ID NOS: 4 to 12; and a third nucleotide sequence encoding an immunomodulator; and a pharmaceutically acceptable carrier.

6. The pharmaceutical composition of claim 5, wherein the immunomodulator is selected from the group consisting of T-cell co-stimulatory factors and cytokines.

7. The pharmaceutical composition of claim 6, wherein the immunomodulator is a cytokine and said cytokine is an interleukin.

8. The pharmaceutical composition of claim 6, wherein the immunomodulator is a T-cell co-stimulatory factor.

9. The pharmaceutical composition of claim 5, wherein the immunomodulator is both a T-cell co-stimulatory factor and a cytokine.

10. The pharmaceutical composition of claim 5, wherein the immunomodulator and the immunogenic MUC1 fragment are encoded on separate viral vectors.

11. The pharmaceutical composition of claim 5, wherein the immunomodulator and the immunogenic MUC1 fragment are encoded on a single viral vector.

12. The recombinant pox virus of claim 5, wherein the pox virus is selected from the group consisting of orthopox, suipox and avipox.

13. The pharmaceutical composition of claim 5, wherein said MUC1 fragment comprises about 6 to 14 tandem repeat units.

14. Use of the pox virus of claim 1 as a first pox virus and a second pox virus selected from the group consisting of orthopox, suipox and avipox for generating an immune response in a mammal having a tumor expressing MUC1.

15. Use of the pox virus of claim 1 as a first pox virus and a second pox virus selected from the group consisting of orthopox, suipox and avipox in the

manufacture of a medicament for generating an immune response in a mammal having a tumor expressing MUC1.

16. The use of any one of claims 14 and 15, wherein the second pox virus is from a viral genus different from said first pox virus.

17. The use of any one of claims 14 and 15, further comprising use of an immunomodulator.

18. Use of the pox virus of claim 4 for generating an immune response in a mammal having a tumor expressing MUC1.

19. Use of the pox virus of claim 4 in the manufacture of a medicament for generating an immune response in a mammal having a tumor expressing MUC1.

20. The recombinant pox virus of claim 1, wherein the pox virus is MVA.

21. The use of claim 17, wherein the immunomodulator is a cytokine or a co-stimulatory molecule.

22. The use of claim 21, wherein said co-stimulatory molecule is B7.

23. The use of claim 22, wherein said B7 is B7.1 or B7.2.

24. The use of claim 21, wherein the cytokine is an interleukin.

25. The use of any one of claims 14 and 15, wherein said first pox virus is selected from the group consisting of an orthopox virus vector, an avipox virus vector, a suipox virus vector, and a capripox virus vector.

26. The use of claim 25, wherein the first pox virus is an orthopox virus.

27. The use of claim 26, wherein the orthopox virus is a vaccinia virus.

28. The use of claim 27, wherein the vaccinia virus is an MVA.

29. The use of any one of claims 14 and 15, wherein the first pox virus is an orthopox virus and the second pox virus is an avipox virus.

30. The use of claim 29, wherein the avipox virus is a fowlpox virus.

31. The use of claim 29, wherein the orthopox virus is a vaccinia virus.

32. The use of claim 31, wherein the vaccinia virus is MVA.

33. The use of any one of claims 14 and 15, wherein said first pox virus further comprises a nucleic acid sequence encoding an immunomodulator.

34. The use of any one of claims 14, 15 and 32, wherein the second pox virus further comprises a nucleic acid sequence encoding an immunomodulator.

35. A recombinant pox virus comprising a nucleic acid sequence encoding an immunogenic MUC1 fragment comprising 5 to 25 MUC1 tandem repeat units, the nucleic acid sequence comprising a first nucleotide sequence having SEQ ID NO: 2; and a second nucleotide sequence comprising 4 to 24 altered nucleotide sequences encoding 4 to 24 altered tandem repeats, wherein each altered tandem repeat is altered from SEQ ID NO: 2 by changing at least one nucleotide of at least one codon of SEQ ID NO: 2 so that the amino acid of SEQ ID NO: 1 is maintained or by substituting at least one codon in SEQ ID NO: 1 such that such substituted codons is selected from the group consisting of substituting at least one of the glycines in SEQ ID NO: 1 to serine, substituting at least one of the serines in SEQ ID NO: 1 to glycine, and substituting the valine in SEQ ID NO: 1 to leucine.

36. A recombinant pox virus comprising a nucleic acid sequence encoding an immunogenic MUC1 fragment comprising 6 identical amino acid tandem repeat units, the nucleic acid sequence comprising a first nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 that is SEQ ID NO: 2; and a second nucleotide sequence encoding 5 copies of the amino acid sequence of SEQ ID NO: 1 as the other 5 tandem repeat units, the second amino acid sequence comprising 5 copies of an

altered nucleotide sequence of SEQ ID NO: 2 by changing wobbled nucleotides of the codons of SEQ ID NO: 2, the 5 copies encoding the other 5 tandem repeat units, and wherein the altered nucleotide sequence is selected from SEQ ID NOS: 4 to 12.

37. A recombinant pox virus comprising nucleic acid sequences encoding 5 to 25 MUC1 tandem repeat units, said tandem repeat units having an amino acid sequence of SEQ ID NO: 1, wherein at least one of the nucleic acid sequences encoding the tandem repeats has SEQ ID NO: 2 and at least one of other nucleic acid sequences encoding the tandem repeats is altered to reduce duplications of codons, wherein the altered nucleotide sequence is selected from SEQ ID NOS: 4 to 12.

38. The recombinant pox virus of claim 37, wherein at least one nucleic acid encoding the tandem repeats is altered by changing wobbled nucleotides of codons of SEQ ID NO: 2.

39. The recombinant pox virus of claim 37, wherein at least one nucleic acid encoding the tandem repeats is altered by the third base of threonine codons 3, 11 and 16 of SEQ ID NO: 2 using ACG, ACT, and ACA respectively.

40. The recombinant pox virus of claim 1 which is rV-MUC1.

41. Use of a MUC1 antigen expressed by the recombinant pox virus of claim 1 for generating MUC1 specific cytotoxic T-lymphocytes.

42. The use of claim 41 for inhibiting a MUC1 positive tumor cell.

43. The use of claim 41 for killing a MUC1 positive tumor cell.

44. A kit containing the pharmaceutical composition of claim 5, and instructions for its use.

45. The kit of claim 44 further containing an adjuvant.

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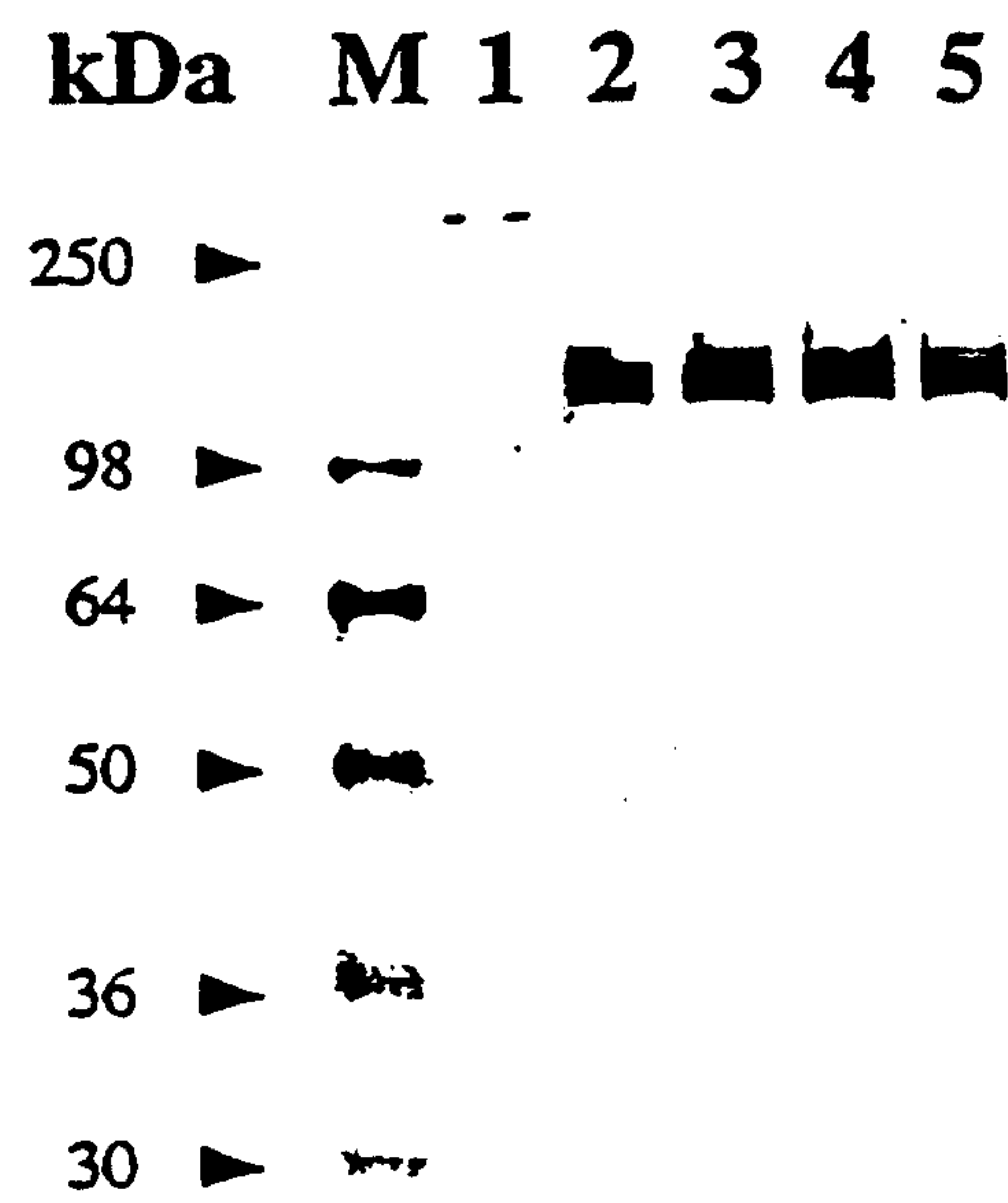


FIGURE 1

FIGURE 2

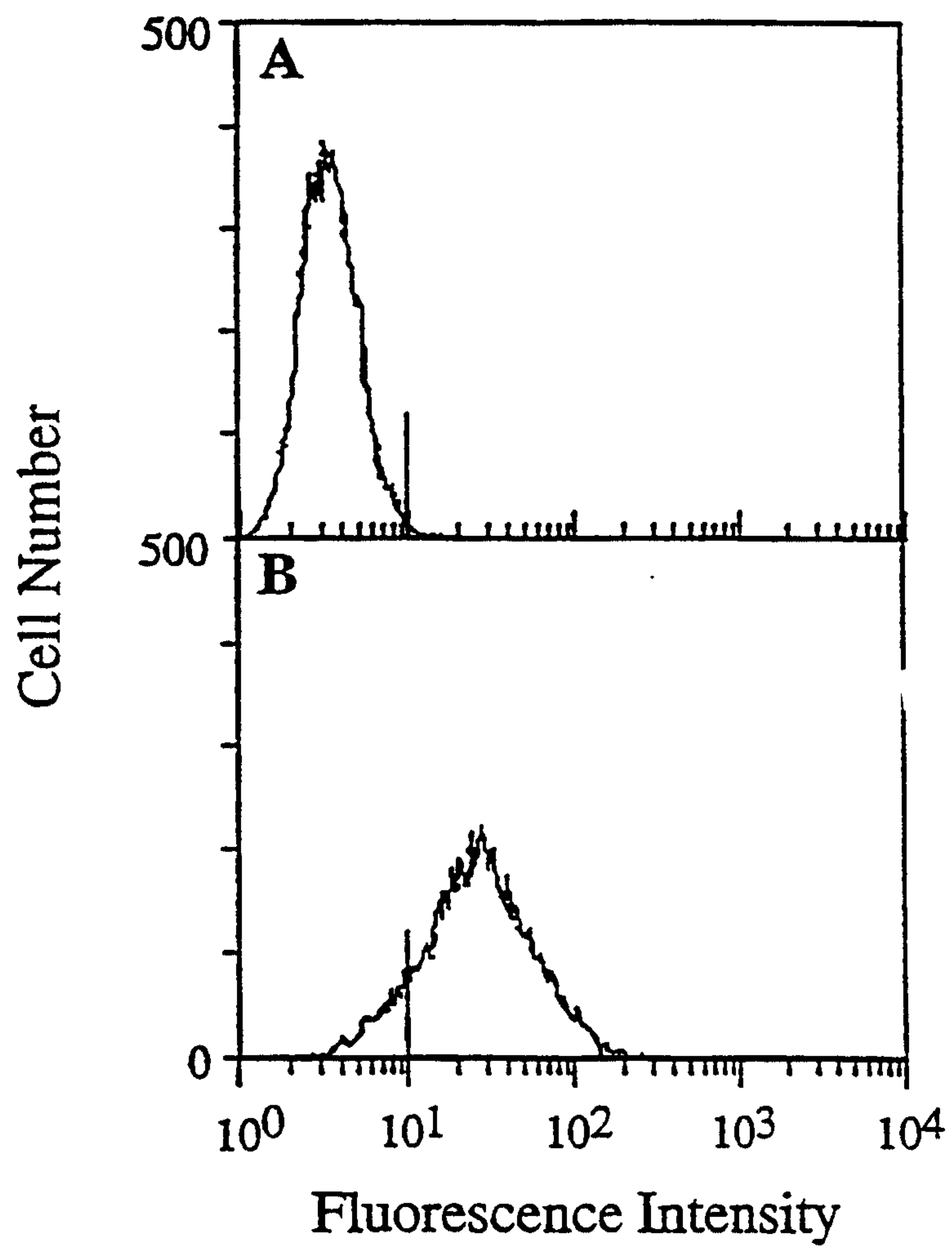


FIGURE 3

