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(54) Titre : POLYPEPTIDE DE FUSION DIRIGE CONTRE UNE TUMEUR INDUITE PAR LE VIRUS EB ET MUTANT DE LA COLICINE IA

(54) Title: FUSION POLYPEPTIDE AGAINST EB VIRUS-INDUCED TUMOR AND COLICIN IA MUTANT

(57) Abrégé/Abstract:
The present invention provides a fusion polypeptide against EB virus-induced tumor, which comprises an antibody or a mimetic antibody against EB virus and an ion channel forming colicin selected from E1, Ia, Ib, A, B, N and their mutants. The present invention also provides a colicin Ia mutant, which comprises mutations of G11A, H22G, A26G, V31L and H40D. The present invention also provides a gene, vector, preparation method and use of the fusion polypeptide, and provides a gene and use of the mutant.

Abstract

The present invention provides a fusion polypeptide against EB virus-induced tumor, which comprises an antibody or a mimetic antibody against EB virus and an ion channel forming colicin selected form E1, Ia, Ib, A, B, N and their mutants. The present invention also provides a colicin Ia mutant, which comprises mutations of G11A, H22G, A26G, V31L, and H40D. The present invention also provides a gene, vector, preparation method and use of the fusion polypeptide, and provides a gene and use of the mutant.

Specification

FUSION POLYPEPTIDE AGAINST EB VIRUS-INDUCED TUMOR AND COLICIN IA MUTANT

FIELD OF THE INVENTION

The present invention relates to the filed of anti-tumor agents, and more specifically, to a novel polypeptide against tumor caused by EB virus and use and preparation method thereof.

RELATED ART

In the area of antibiotics research, studies have been directed towards development of new antibiotics which simulate the inter-killing mechanism among homogeneous heterologous strains. There are a lot of bacterial toxins in the nature which kill cells by forming ion channels on the cellular membrane of bacteria directly. The model example of such toxin is colicin, a bacteria toxin secreted by *E. coli*. Colicin Ia was found by Jacob in 1952, since then, via the hard work of generations, Qiu et al. (Major transmembrane movement associated with colicin Ia channel gating. J. Gen. Physiology, 107:313-328 (1996)) finally revealed the transmembrane spatial structure of colicin Ia when the ion channels formed in artificia lipid bilayer membranes is open or closed, which provides a fundamental basis for the design and preparation of new antibiotics at molecular level. Subsequently, there are polypeptide molecules made by the connection of colicin polypeptide with signal peptide such as pheromones of *Streptococcus albus* or *Staphylococcus*, which target the colicin to the cell membrane of bacteria interested and kill the cell due to the leak of cellular contents through the transmembrane ion channels formed.

Malignant tumor poses a great threat to human health. Seven million people die from malignant tumor every year in the world, one sixth of which are in China. Malignant tumor is now the second leading cause of death in our country. Since the etiology, pathogenesis and clinical manifestation of malignant tumor are not clearly elucidated, prevention and treatment is not effective. Anti-tumor agents are important in the treatment of tumor. Although they achieve therapeutic effect to some tumor, there remains some disadvantage, such as insufficient tumor selectivity, immunological suppression, adverse reaction, drug resistance, etc.

The surface of cells of Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal

carcinoma caused by Epstein-Barr (EB) Virus bears a specific surface antigen of EB virus. Therefore, EB virus surface antigen can be regarded as a specific marker of such tumor cells. For the agents against the tumor caused by EB virus, the invention with the china patent No. ZL200410081446.8 discloses an anti-tumor polypeptide formed by the conjugation of colicin and antibody mimetics which recognize EB virus surface antigen. The anti-tumor polypeptide can specifically kill the cancer cells caused by EB virus in the body, has no harm to normal cells, the killing ability of which is several times over other anti-tumor agents, and overcomes the problems such as tumor selectivity, drug resistance, impairment of normal tissue when the cancer cells are killed. Xiao-Qing Qiu et al. (Xiao-Qing Qiu et al., 2007, Small antibody mimetics comprising two complementarity-determining regions and a framework region for tumor targeting, *Nature Biotechnology* 25, 921–929, 1 August 2007) compare the killing effect of anti-tumor polypeptides constructed by a series of antibody mimetics and colicin, and find that anti-tumor polypeptides constructed by antibody mimetics of V_HCDR1-V_HFR_2-V_LCDR3 and V_LCDR1-V_HFR_2-V_HCDR3 with colicin have superior killing ability. This work provides more candidate antibody mimetics for the preparation of polypeptides against tumor caused by EB virus.

However, for the anti-tumor polypeptide described above, since the hydrophobic terminal of colicin has some amino acid residues which may include hypersensitivity, the medicine comprising polypeptide of colicin is possible to elicit abnormal immune responses in vivo more easily. It's reported that metabolic mechanism of many cancer patient is abnormal due to the disturbance from cancer cells, they are easy to suffer an allergic response to medicine of polypeptides, thus can not be treated by such medicine. Therefore, it is necessary to improve colicin polypeptide in order to obtain an anti-cancer medicine which is safer and suitable for more patients.

SUMMARY OF THE INVENTION

Base on the disadvantage of prior art stated above, the present invention provides a novel polypeptide against tumor caused by EB virus and use and preparation method thereof, thus provides a medicine for the treatment of tumor caused by EB virus which has high killing ability, high specificity, and low possibility of allergy.

A novel polypeptide against tumor caused by EB virus, which is formed by operable linkage of a mutant polypeptide of colicin which can form ion channels with a polypeptide of anti-EB

virus antibody or a polypeptide of anti-EB virus antibody mimetics, the mutant polypeptide of colicin which can form ion channels is obtain by mutation of amino acid residues of G11A, H22G, A26G, V31L, and H40D to peptide chain of wild-type colicin E1, Ia, Ib, A, B, N or aqueous channel domain thereof, the amino acid sequence of the polypeptide of anti-EB virus antibody is the same as the polypeptide of monoclonal antibody secreted by hybridoma of ATCC HB-168.

The polypeptide of antibody mimetics is a connected peptide of CDR1 region of heavy chain, linking peptide segment of CDR1-CDR2 of heavy chain and CDR3 of light chain of anti-EB virus antibody.

The mutant polypeptide of colicin which can form ion channels is obtained by mutation of wild-type colicin Ia.

The novel polypeptide against tumor caused by EB virus has the amino acid sequence shown in SEQ ID NO. 29.

A gene encoding the novel polypeptide against tumor caused by EB virus.

The gene, which has the nucleotide sequence shown in SEQ ID NO. 30.

A recombination plasmid comprising said gene.

A preparation method for the novel polypeptide against tumor caused by EB virus, comprising steps of: transforming said recombination plasmid into an expression system for expression, and isolating the polypeptide expressed.

Use of said novel polypeptide against tumor caused by EB virus in preparation of a medicament for the treatment and prevention of tumor caused by EB virus.

A mutant polypeptide of colicin Ia, its amino acid sequence is shown in SEQ ID NO. 24.

A gene encoding a mutant polypeptide of colicin Ia.

Use of said gene in preparation of peptide medicament, operably linking said gene with a gene which induces the peptide, cloning into an expression vector, then transforming the expression vector into an expression system, and isolating the polypeptide expressed.

The invention provides a novel polypeptide against tumor caused by EB virus, which is formed by a mutant polypeptide of colicin which can form ion channels with a polypeptide of anti-EB virus antibody or a polypeptide of anti-EB virus antibody mimetics. Since there are some amino acid residues in the wild-type colicin polypeptide molecule which may include hypersensitivity, in the polypeptide molecule of colicin which can form ion channel construct, the invention selectively mutates amino acid residues in the hydrophobic region which may elicit

allergic response easily. For example, in a preferred embodiment of the invention, the mutant sites of polypeptide of colicin 1a are: G11A, H22G; A26G; V31L and H40D. In mice immunized with injection of a polypeptide of colicin 1a or a polypeptide of mutant 1a respectively, the experimental data shows that serum titer produced by the mice injected with the polypeptide of mutant 1a is several orders of magnitude lower than the former, that is to say, the level of immune response is lower, demonstrating that the mutant polypeptide reduces the possibility of allergy, while the mutant polypeptide retains the function of forming ion channels in cell membrane. The experiment showed that the killing ability of the recombinant polypeptide of the invention is not affected, which means that the mutant amino acid residues do not affect the function of forming ion channels for colicin. In the novel polypeptide against tumor caused by EB virus provided by the invention, via the recognition of the polypeptide of anti-EB virus antibody or the polypeptide of anti-EB virus antibody mimetics to the surface antigen of tumor cells caused by the EB virus, the mutant polypeptide of colicin is targeted to the membrane of target cells, the hydrophobic region of transmembrane ion channel domain of the mutant polypeptide of colicin is inserted to the cell membrane of tumor cells, and forms an ion channel, therefore the tumor cells die from the leak of cellular contents. The amino acid sequence of polypeptide of anti-EB virus antibody completely refers to the amino acid sequence of the polypeptide of antibody secreted by hybridoma of ATCC HB-168.

In an embodiment of the invention, an anti-tumor polypeptide of low molecular weight of the invention is preferred, which is obtained by operable linkage of the polypeptide of anti-EB virus antibody mimetics described above with the carboxyl terminus of the mutant polypeptide of colicin. That is to say, such a mimetic polypeptide of low molecular weight comprises a peptide chain of VHCDR1-VHFR2-VLCDR3 which is obtained by the connection of VHCDR! region, VLCDR3 region, linking peptide segment of VHCDR1-VHCDR2 and VLCDR3 of light chain of the polypeptide of the anti-EB virus antibody. The amino acid sequence of the novel anti-tumor peptide 1 of antibody mimetics is shown in SEQ ID NO. 25. The antibody mimetics only comprises amino acids less than 30, and has a much lower molecular weight than natural antibody of 150 amino acids. It fulfills the requirement of antigen recognition while reduces the molecular weight of anti-tumor polypeptide substantially, and contributes to the tissue penetration ability of the anti-tumor polypeptide of the present invention.

According to an aspect of the invention, there is provided a polypeptide for treating tumors caused by EB virus, said polypeptide being formed by operable linkage of a mutant polypeptide of colicin which can form ion channels with a polypeptide of anti-EB virus antibody or a polypeptide of anti-EB virus antibody mimetics, the mutant polypeptide of colicin which can form ion channels is obtained by mutation of amino acid residues of G11A, H22G, A26G, V31L, and H40D to peptide chain of wild-type colicin 1a, wherein the polypeptide of anti-EB virus antibody is the monoclonal antibody secreted by hybridoma of ATCC HB-168 and the polypeptide of antibody mimetics comprises: CDR1 heavy chain of anti-EB virus antibody; CDR1-CDR2 heavy chain linking segment of anti-EB virus antibody; and CDR3 light chain of anti-EB virus antibody.

According to another aspect of the invention, there is provided a nucleic acid encoding the polypeptide against tumors caused by EB virus as described above.

According to yet another aspect of the invention, there is provided a recombination plasmid comprising the nucleic acid described above.

According to a further aspect of the invention, there is provided a preparation method for the polypeptide against tumor caused by EB virus as described above, comprising steps of: transforming the recombination plasmid described above into an expression system for expression, and isolating the polypeptide expressed.

According to a still further aspect of the invention, there is provided use of the polypeptide against tumor caused by EB virus as described above in preparation of a medicament for the treatment and prevention of tumor caused by EB virus.

According to yet another aspect of the invention, there is provided a mutant colicin 1a polypeptide, its amino acid sequence is shown in SEQ ID NO. 24.

According to a further aspect of the invention, there is provided a nucleic acid encoding the mutant colicin 1a polypeptide as described above.

According to another aspect of the invention, there is provided use of the polypeptide prepared according to the method of claim 6 for treating tumors caused by EB virus.

Another aspect of the present invention is to provide a gene sequence encoding the

anti-tumor polypeptide of the present invention. The gene of the anti-tumor polypeptide of the present invention is formed by the operable linkage of a gene encoding a mutant polypeptide of colicin with a gene encoding a polypeptide of anti-EB virus antibody or a polypeptide of antibody mimetics thereof, wherein the polypeptide of colicin and the gene sequence of the anti-EB virus antibody is known in the art, the gene of mutant polypeptide of colicin is obtained by the following point mutations in the corresponding codons of the gene of colicin polypeptide: G11A, H22G, A26G, V31L and H40D. As a result of the degeneracy of the genetic code, a skilled person in the art may adjust the nucleotide sequence encoding the anti-tumor polypeptide of the present invention without altering the amino acid sequence.

The recombination plasmid of the present invention means that the original vector loaded with gene of wild-type colicin is site-directed mutated in double stranded nucleotide, and inserted by mutant codons in the site of target mutation, thus obtaining a mutant vector comprising the gene of mutant polypeptide of colicin. The same process of the site-directed mutagenesis inserts a gene encoding antibody mimetics of an anti-EB virus antibody into the carboxyl terminus of a gene of the mutant polypeptide of colicin, thus obtaining a recombinant plasmid of the present invention. The original vector pSELECTTM-1 is purchased from Promega Corp., which carries genes of colicin Ia and Immunity protein. The process of site directed mutagenesis follows the instruction of the kit from Strategene Corp. The present invention carries out some site directed mutagenesis to prepare a mutant polypeptide of colicin, wherein five codons are site-directed mutated. Therefore, 5 pairs of primer sequences are designed (SEQ ID No. 1-10). In the example of the present invention, 6 pairs of primer sequences are designed for the gene of antibody mimetics (SEQ ID No. 11-22).

The present invention also provides a method for the preparation of the anti-tumor polypeptide of the present invention, which comprises transforming the recombinant vector obtained above into an engineering bacteria of *E. coli* BL21(DE3), selecting positive clone, isolating and purifying the protein expressed by the positive clone, thus obtaining the novel polypeptide against tumor caused by EB virus of the present invention.

The novel polypeptide against tumor caused by EB virus provided by the present invention can be used in preparation of a medicament for the treatment and prevention of tumor caused by EB virus. A clinical suitable pharmaceutical composition can be made by adding the polypeptide of novel antibiotics obtain in the present invention into a pharmaceutically acceptable carrier or

vehicle or other optional components.

The present invention also provides the amino acid sequence and the gene sequence of the mutant polypeptide of colicin Ia. The mutant polypeptide can be used in the present invention, also can be used in the construction of an antibody polypeptide with other targeting polypeptides. The experimental data of example 3 in the invention proves that the peptide medicament comprising the mutant polypeptide has a low immunogenicity, and that the antibody polypeptide formed by the mutant polypeptide with other targeting polypeptide has a bactericidal ability. The preparation method is routine experimental process in the art.

The novel anti-tumor polypeptide provided by the invention has the advantage of the anti-tumor polypeptide disclosed in the patent No. ZL200410081446.8, i.e., highly specific targeting and safety to normal cells, and not inclined to developing drug resistance. At the same time, the anti-tumor polypeptide of the present invention has been mutated at the amino acid residues which tend to elicit allergic response, the immunogenicity of the anti-tumor polypeptide comprising such mutant polypeptide is reduced, that is to say, the possibility of allergic reaction is reduced. The use safety and effect of killing tumor of medicament of such polypeptides are improved. This may also be a good example for improvement of other medicament comprising colicin polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic illustration of the structure of recombinant plasmid pCHCEB11 which comprises the gene of polypeptide of antibody mimetics of V_HCDR1-V_HFR_2-V_LCDR3 and the gene of the mutant polypeptide of colicin Ia.

Figure 2. Schematic illustration of the structure of recombinant plasmid pCHCEB22 which comprises the gene of polypeptide of antibody mimetics of V_HCDR1-V_HFR_2-(Rev)V_LCDR3 and the gene of the mutant polypeptide of colicin Ia.

Figure 3. The experiment 1 of sensitization effect of the mutant polypeptide of colicin Ia.

(A) Kunming mice intraperitoneally injected with lethal dose of MRSA (ATCC BAA42) are grouped randomly into (1) control group, (2) group of ampicillin , (3) group of polypeptide against *S. aurous* (ZL 01128836.1), (4) group of polypeptide 1 against *S. aurous*.

(B) After 14 days, a new batch of Kunming mice are grouped into a control group and a group of ampicillin. Survived mice from the group of polypeptide against *S. aurous* and the group

of polypeptide 1 against *S. aurous* are grouped into a group of polypeptide against *S. aurous* and a group of polypeptide 1 against *S. aurous*, and the experiment is repeated.

(C) After 41 days, a new batch of Kunming mice are grouped into (1) control group, (2) group of levofloxacin, (3) group of ceftriaxone sodium, (4) group of polypeptide against *S. aurous*, and (5) survived mice from the group of polypeptide 1 against *S. aurous* are grouped into a group of polypeptide 2 against *Pseudomonas aeruginosa*, and a group of polypeptide 1 against *Pseudomonas aeruginosa*.

Figure 4. The experiment 2 of low sensitization effect of the mutant polypeptide of colicin Ia.

(A) the serum of group of polypeptide against *S. aurous*/polypeptide 2 against *Pseudomonas aeruginosa*, titer of 1:50,000 ;

(B) the serum of group of polypeptide 1 against *S. aurous*/polypeptide 1 against *Pseudomonas aeruginosa*, titer of 1:50,000.

(1) Week 1, (2) Week 2, (3) serum of Week 7, (4) negative control.

Figure 5. Comparison of in vitro killing effect of the novel anti-tumor polypeptide to Burkitt's lymphoma caused by EB virus.

(A) control group, (B) novel anti-tumor polypeptide 1 treated group, (C) novel anti-tumor polypeptide 2 treated group.

Figure 6. In vitro killing effect of the novel anti-tumor polypeptide to cells of Burkitt's lymphoma caused by EB virus and other tumor cells.

(A) EBV positive cells of Burkitt's lymphoma,

(B) EBV negative cells of Burkitt's lymphoma,

(C) EBV positive cells of malignant lymphosarcoma from patient of AIDS.

(1) control group, (2)novel anti-tumor polypeptide 1 treated group.

Figure 7. Killing effect of the novel anti-tumor polypeptide to solid tumor grown from naked mice planted with cells of Burkitt's lymphoma caused by EB virus.

(A) control group.

(B) SCID immunodeficient mice from the novel anti-tumor polypeptide 1 treated group are all inoculated with cells of Burkitt's lymphoma into both axillary flanks. Arrow on the left, EBV

negative lymphosarcoma, arrow on the right, EBV positive lymphosarcoma.

Figure 8. Killing effect of the novel anti-tumor polypeptide to solid tumor grown from naked mice planted with cells of Burkitt's lymphoma caused by EB virus.

(A) section of EBV negative lymphosarcoma of control mice, (B) section of EBV positive lymphosarcoma of control mice, (C) section of EBV negative lymphosarcoma of novel anti-tumor polypeptide 1 treated mice, (D) section of EBV positive lymphosarcoma of novel anti-tumor polypeptide 1 treated mice.

EMBODIMENTS

The invention will now be describe by describing preferred embodiment of the invention and with reference to the accompany drawings.

The original vector pSELECT^{TM}-1 used in the invention is purchased from Promega Corp..

The engineering bacteria of *E. coli* BL21(DE3) is purchased from Novagen Corp..

Example 1. Construction of recombinant plasmid comprising gene encoding mutant colicin Ia.

The original vector is the plasmid pSELECTTM-1 (8.3 kb) (purchased from Promega Corp.) which carries genes of colicin Ia and Immunity protein. Sequences of oligonucleotide primers shown in SEQ ID NOs.1-10 which encode mutant amino acids is operably linked to the gene of wild-type colicin Ia respectively by a double-strand oligonucleotide Site-Directed Mutagenesis technology (QuickChangeTM Kit, Strategene Corp.), obtaining a gene shown in SEQ ID NO.23 which encodes a mutant polypeptide of colicin Ia, and a mutant plasmid. After that, the gene of SEQ ID NO.26 or SEQ ID NO.28 is inserted into the mutant plasmid after the codon of I626 of gene of mutant polypeptide of colicin Ia, obtaining two recombinant plasmids pCHCEB11 (shown in Figure 1) and pCHCEB22 (shown in Figure 2) for the novel polypeptide against tumor caused by EB virus. Sequences of 6 pairs of oligonucleotide primers are shown in SEQ ID NO.11-22, which are designed for the preparation of gene encoding the antibody against EB virus in the recombinant plasmid. The recombination plasmid is transfected into the engineering bacteria of *E. coli* BL21(DE3) (purchased from Novagen Corp.) to prepare the polypeptide. The polypeptides obtained are shown in SEQ ID NO.29 (hereinafter referred to as "novel anti-tumor polypeptide 1") and SEQ ID NO.31 (hereinafter referred to as "novel anti-tumor polypeptide 2") in the sequence

list.

The process of double-strand oligonucleotide site-directed mutagenesis follows the Strategene QuickChang Site-Directed Mutagenesis Kit (catalog#200518).

1. Preparation of reactant for site-directed mutagenesis:

5 μl 10X buffer

2 μl (10 ng) original plasmid pSELECT^{TM}-1 which carries genes of polypeptide of wild-type of colicin Ia and Immunity protein.

1.25 μl (125 ng) 5'-3' oligonucleotide primer designed

1.25 μl (125 ng) 3'-5' oligonucleotide primer designed

1 μl dNTP

double-distilled water 50 μl

1 μl pfu

(provided by the Kit except the plasmid, primers and double-distilled water)

2. PCR amplification, amplification condition: 25 cycles of denaturation at 95°C for 35 seconds, anneal at 53°C for 70 seconds, and extension at 68°C for 17 minutes;

3. 1 μl endonuclease Dpn 1 is added to digest parental DNA chain (37°C, 1 hour), 1 μl reactant and 50 μl XL1-Blue competent cell are incubated together on ice for 30 minute, after a heat shock at 42°C for 45 second, incubated in ice for 2 minute;

4. 0.5 ml cultivation medium NZY is added, shaking at 37°C and 220 rpm for 1 hour. 50-100 μl reactant is plated (LB medium plus 1% agar and 50 μg/ml ampicillin, overnight at 37°C);

5. Colony is picked up after 18 hours. Plasmid is extracted, sequenced, confirming that the mutation is successful;

6. The 50 ng recombination plasmid obtained finally by mutation at multiple sites is incubated with 40 μl *E. coli* BL-21(DE3) competent cells on ice for 5 minute, heat shocked at 42°C for 30 second, and incubated in ice for 2 minute. 160 μl cultivation medium SOC from Novagen crop. is added, and plated after shaking at 37°C and 220 rpm for 1 hour (LB medium plus 1% agar and 50 μg/ml ampicillin, overnight at 37°C).

7. Single colony is picked up for amplification, 8-16 liters FB medium, 250 rpm, 30°C for 4-5 hours, heat shocked at 42°C and 250 rpm for 30 minute, and at 37°C for 2 hours. The bacterium is precipitated by centrifugation at 6000g and 4°C for 20 minute. 50 mM borate buffer (2mM EDTA +2mM DTT) at 4°C and 50-80 ml bacterium suspension is added with 0.2M PMSF 250 ml and

treated with ultrasonication (4°C, 400W, 2 minutes). Bacterium debris is high speed centrifugated (4°C, 75,000g, 90 minutes). The supernatant is added with 5 million units of streptomycin sulphate to precipitate DNA. After precipitation by centrifugation at 15000g and 4°C for 10 minutes, the supernatant is dialysed overnight in dialysis bag of molecular weight 15,000 in 50 mM borate buffer at 4°C. After precipitation by centrifugation at 15000g and 4°C for 10 minutes again, the supernatant is loaded on a CM ion-exchange column. The column is eluted using a gradient of 0.1-0.3 M NaCl + 50 mM borate buffer, obtaining the recombinant anti-tumor polypeptide.

Sequences of primers designed for site-directed mutagenesis are as follows:

SEQ ID NO.1, oligonucleotide primer 5'-3'designed for mutation of G11A in gene of colicin:

cgt att aca aat ccc GCA gca gaa tcg ctg ggg

SEQ ID NO.2, oligonucleotide primer 3'-5' designed for mutation of G11A in gene of colicin:

ccc cag cga ttc tgc TGC ggg att tgt aat acg

SEQ ID NO.3, oligonucleotide primer 5'-3' designed for mutation of H22G in gene of colicin:

gat tca gat ggc GGT aaa tta tgg gtg

SEQ ID NO.4, oligonucleotide primer 3'-5' designed for mutation of H22G in gene of colicin:

cac cca taa ttt ACC gcc atc tga atc

SEQ ID NO.5, oligonucleotide primer 5'-3' designed for mutation of A26G in gene of colicin:

gaaa ttatgGGTgt tgatatttat

SEQ ID NO.6, oligonucleotide primer 3'-5' designed for mutation of A26G in gene of colicin:

ataaatatacaacACCcataatttc

SEQ ID NO.7, oligonucleotide primer 5'-3' designed for mutation of V31L in gene of colicin:

gt tgatatttat CTC aaccctc cacgtgtc

SEQ ID NO.8, oligonucleotide primer 3'-5' designed for mutation of V31L in gene of colicin:

gacacgtggagggttGAGataàatatcaac

SEQ ID NO.9, oligonucleotide primer 5'-3' designed for mutation of H40D in gene of colicin:

cgtgtcga tgtctttGATggtaccccgc ctgcat

SEQ ID NO.10, oligonucleotide primer 3'-5' designed for mutation of H40D in gene of colicin:

atgcaggcggggtaccATCaaagacatcgacacg

SEQ ID NO.11, primer 5'-3' for gene of V_HCDR1 in recombination plasmid pCHCEB11:

gcg aat aag ttc tgg ggt att TCC TTC GGT ATG CAT TGG GTG CGTCAGtaa ata aaa tat aag aca ggc

SEQ ID NO.12, primer 3'-5' for gene of V_HCDR1 in recombination plasmid pCHCEB11:

gcc tgt ctt ata ttt tat tta CTG ACG CAC CCA ATG CAT ACC GAA GGA aat acc cca gaa ctt att cgc

SEQ ID NO.13, primer 5'-3' for gene of V_HFR_2 in recombination plasmid pCHCEB11:

ggt atg cat tgg gtg cgt cag GCC CCC GAG AAA GGT CTG GAG TGG GTG GCC taa ata aaa tat aag aca ggc

SEQ ID NO.14, primer 3'-5' for gene of V_HFR_2 in recombination plasmid pCHCEB11:

gcc tgt ctt ata ttt tat tta GGC CAC CCA CTC CAG ACCT TTT CTC GGG GGC ctg acg cac cca atg cat acc

SEQ ID NO.15, primer 5'-3' for gene of (Rev)V_lCDR3 in recombination plasmid pCHCEB11:

aaa ggt ctg gag tgg gtg gcc ACC TAC CCC TAC TCC TAC GGT CAG GGT taa ata aaa tat aag aca ggc

SEQ ID NO.16, primer 3'-5'for gene of (Rev)V_LCDR3 in recombination plasmid pCHCEB11:

gcc tgt ctt ata ttt tat tta ACC CTG ACC GTA GGA GTA GGG GGT ggc cac cca ctc cag acc ttt

SEQ ID NO.17, primer 5'-3' for gene of V_HCDR1 in recombination plasmid pCHCEB22:

gcg aat aag ttc tgg ggt att TCC TTC GGT ATG CAT TGG GTG CGT CAG taa ata aaa tat aag aca ggc

SEQ ID NO.18, primer 3'-5' for gene of V_HCDR1 in recombination plasmid pCHCEB22:

gcc tgt ctt ata ttt tat tta CTG ACG CAC CCA ATG CAT ACC GAA GGA aat acc cca gaa ctt att cgc

SEQ ID NO.19, primer 5'-3' for gene of V_HFR_2 in recombination plasmid pCHCEB22:

ggt atg cat tgg gtg cgt cag GCC CCC GAG AAA GGT CTG GAG TGG GTG GCC taa ataaaa tat aag aca ggc

SEQ ID NO.20, primer 3'-5' for gene of V_HFR_2 in recombination plasmid pCHCEB22:

gcc tgt ctt ata ttt tat tta GGC CAC CCA CTC CAG ACCT TTT CTC GGG GGC ctg acg cac

cca atg cat acc

SEQ ID NO.21, primer 5'-3' for gene of V_LCDR3 in recombination plasmid pCHCEB22:

aaa ggt ctg gag tgg gtg gcc GGT CAG GGT TAC TCC TAC CCC TAC ACC taa ata aaa tat aag aca ggc

SEQ ID NO.22, primer 3'-5' for gene of V_LCDR3 in recombination plasmid pCHCEB22:

gcc tgt ctt ata ttt tat tta GGT GTA GGG GTA GGA GTA ACC CTG ACC ggc cac cca ctc cag acc ttt

Example 2. Observation of immune effect of novel anti-tumor polypeptides prepared from recombination plasmid pCHCEB11 and pCHCEB22.

Mice are immunized with the novel anti-tumor polypeptide 1 and the novel anti-tumor polypeptide 2 prepared from the recombination plasmid pCHCEB11 and pCHCEB22 obtained in Example 1, and the anti-tumor polypeptide 1 and the anti-tumor polypeptide 2 from the former invention owned by inventor (ZL200410081446.8). Each protein described above is mixed with adjuvant. The priming dose and the boost dose are one intraperitoneal injection of 50 μg (0.5 ml) each mouse, five injections with 2 weeks interval totally. Serum titer is determined by indirect ELISA method. The titer of mice immunized with the novel anti-tumor polypeptide 1 and 2 prepared by the present invention range from 10^{-3} to 10^{-4}, while the titer of mice immunized with the anti-tumor polypeptide 1 and anti-tumor polypeptide 2 range from 10^{-4} to 10^{-5}.

It can be seen that the possibility of hypersensitive reaction induced by the novel anti-tumor polypeptide of the present invention is 1 order to 2 orders of magnitude lower than the possibility of hypersensitive reaction induced by anti-tumor polypeptide comprising wild-type colicin Ia.

Example 3. Experiment of low sensitization effect of the mutant polypeptide of colicin Ia which forms novel anti-tumor polypeptide.

The mutant plasmid for mutant polypeptide of colicin Ia (which is mutated at amino acid residues of G11A, H22G, A26G, V31L, and H40D in peptide chain of aqueous channel domain) of Example 1 is operably linked to pheromone AgrD1(YSTCDFIM) of *S. aurous* at N-terminus or C-terminus of the mutant polypeptide, obtaining two antibacterial polypeptides. The polypeptide obtained by the linkage of AgrD1 at carboxyl terminus of the mutant colicin Ia is named as polypeptide 1 against *S. aurous*, and the polypeptide obtained by the linkage of AgrD1 at amino

terminus of the mutant colicin Ia is named as polypeptide 1 against *Pseudomonas aeruginosa.* Plasmid for wild-type colicin Ia is linked at amino terminus to pheromone AgrD1(YSTCDFIM) of *S. aurous*, obtaining polypeptide 2 against *Pseudomonas aeruginosa.*

Experiment 1: A batch of Kunming mice are intraperitoneally injected with lethal dose of MRSA (ATCC BAA42), and are grouped randomly into (1) control group, (2) group of ampicillin , (3) group of polypeptide against *S. aurous* , (4) group of polypeptide 1 against *S. aurous*. Each group consists of 10 mice.

Treating method:

One hour after intraperitoneal injection of lethal dose of MRSA (ATCC BAA42):

control group: injected with 0.5 ml 0.3 M NaCl + 50 mM borate buffer via tail vein once;

group of ampicillin: injected with ampicillin of 2.5 mg/kg via tail vein once;

group of polypeptide against *S. aurous*: injected with polypeptide against *S. aurous* owned by the inventor (ZL 01128836.1) of 6 mg/kg via tail vein once;

group of polypeptide 1 against *S. aurous*: injected with polypeptide 1 against *S. aurous* of 6 mg/kg via tail vein once;

Result: Mice in the control group and the group of ampicillin are all dead in two days. 85% mice in the group of polypeptide against *S. aurous* and the group of polypeptide 1 against *S. aurous* survive.

Experiment 2: 14 days after experiment 1, a new batch of Kunming mice are grouped into a control group and a group of ampicillin. The survived mice from the group of polypeptide against *S. aurous* and the group of polypeptide 1 against *S. aurous* are grouped into a group of polypeptide against *S. aurous* and a group of polypeptide 1 against *S. aurous* to repeat the experiment described above. Mice in the control group and the group of ampicillin are all dead in two days. 75% mice in the group of polypeptide against *S. aurous* survive, and 90% mice in the group of polypeptide 1 against *S. aurous* survive.

Experiment 3: 41 days after experiment 1, a new batch of Kunming mice are grouped into a control group, a group of levofloxacin, and a group of ceftriaxone sodium. The survived mice from the group of polypeptide against *S. aurous* and the group of polypeptide 1 against *S. aurous*

are grouped into a group of polypeptide 2 against *Pseudomonas aeruginosa*, and a group of polypeptide 1 against *Pseudomonas aeruginosa*.

Mice are intraperitoneally injected with of lethal dose of multi-drug resistance *Pseudomonas aeruginosa* (clinical isolates 13578 from Department of Experimental Medicine, West China Hospital of Sichuan University). After one hour,

the control group are injected with 0.5 ml 0.3 M NaCl + 50 mM borate buffer via tail vein once;

the group of levofloxacin are injected with levofloxacin of 5 mg/kg via tail vein once;

the group of ceftriaxone sodium are injected with ceftriaxone sodium of 30 mg/kg via tail vein once;

the group of polypeptide 2 against *Pseudomonas aeruginosa* are injected with the polypeptide 2 against *Pseudomonas aeruginosa* of 8 mg/kg via tail vein once;

the group of polypeptide 1 against *Pseudomonas aeruginosa* are injected with the polypeptide 2 against *Pseudomonas aeruginosa* of 8 mg/kg via tail vein once.

Mice in the control group and the group of levofloxacin are all dead in a day. 25% mice in the group of ceftriaxone sodium survive. 60% mice in the group of polypeptide 2 against *Pseudomonas aeruginosa* survive. All of the mice in the group of polypeptide 1 against *Pseudomonas aeruginosa* survive. It is demonstrated that the antibody of host interfere with the killing effect of the mutant polypeptide lower than with that of wild-type polypeptide.

See figure 3.

At week 1, week 2 and week 7 of the experiment, serum of survived mice from the group of polypeptide against *S. aurous*/group of polypeptide 2 against *Pseudomonas aeruginosa*, and the group of polypeptide 1 against *S. aurous*/group of polypeptide 1 against *Pseudomonas aeruginosa* is assayed by indirect ELISA method to detect the antibody in blood. Wells of enzyme label plate are coated with wilt-type colicin Ia and the mutant polypeptide of colicin Ia, 100 ng/ well. The first antibodies are serums of survived mice from the group of polypeptide against *S. aurous* /group of polypeptide 2 against *Pseudomonas aeruginosa*, and the group of polypeptide 1 against *S. aurous*/group of polypeptide 1 against *Pseudomonas aeruginosa*. The second antibody is goat anti mouse labeled antibody. The first antibody of negative control is 5% milk-PBS. The results of 1:50,000 titer are as follows (see figure 4):

	A(group of polypeptide against *S. aurous* /group of polypeptide 2 against *Pseudomonas aeruginosa*)	(group of polypeptide 1 against *S. aurous*/group of polypeptide 1 against *Pseudomonas aeruginosa*
1(Week 1)	0.914	0.254
2(Week 2)	1.623	0.598
3(Week 7)	2.911	1.41
4(controll)	0.065	0.069

It is demonstrated that the possibility of host's hypersensitive reaction induced by the mutant polypeptide of colicin Ia prepared by the present invention is lower than the possibility of host's hypersensitive reaction induced by wild-type colicin Ia.

Example 4. In vitro killing effect of the novel anti-tumor polypeptide to Burkitt's lymphoma caused by EB virus.

EBV positive cell strain and EBV negative cell strain is standard cell strain from ATCC, USA.

Cell cultivation: 0.1 ml suspension of revived Raji cell is added slowly into 3 ml 1640 liquid medium (plus 10% serum) in a culture dish (dilution rate, 1:30), mixed, and cultured in a 37°C incubator with CO_2. The EBV positive cell strain is ATCC CCL-86 (a standard Burkitt's lymphoma cell used in laboratories in the world, Raji cell, isolated from a 12 year old Africa boys in 1963).

The test cells are grouped into 3 groups.

The group 1 is a blank group, which is added with a preservation solution (10mMPB+0.2M NaCl phosphate buffer (pH7.4)) without the anti-tumor polypeptide.

The group 2 is added with 200μg/ml the novel anti-tumor polypeptide 1 (plasmid pCHCEB11, preservation solution, 10mMPB + 0.2M NaCl phosphate buffer, pH7.4).

The group 3 is added with 200μg/ml the novel anti-tumor polypeptide 2 (plasmid pCHCEB22, preservation solution, 10mMPB + 0.2M NaCl phosphate buffer, pH7.4).

After cultivation for 24 hours, the culture dish is added with the treating agents described above. 72 hours after the addition of the treating agents, the culture dish is added with 20 μl of 100 μMol propidium iodide (PI) , and observed under microscope 10 minutes later. The result shows that the cells of blank group grow well, and the most of cells in the group of novel

anti-tumor polypeptide 1 are stained red by PI, showing that cell membrane is destroyed by the anti-tumor polypeptide, which leads to the death of tumor cells. Comparing the number of dead cells, the effect of novel anti-tumor polypeptide 2 is not so well among two novel anti-tumor polypeptides, see figure 5.

Example 5. Observation of multi-fluorescence staining for the in vitro killing effect of the novel anti-tumor polypeptide to cells of Burkitt's lymphoma caused by EB virus and other tumor cells.

The condition of cell cultivation is the same as Example 2. Three cell strains are used in the experiment: EB-virus positive cell strain: ATCC CCL-86(Raji cell, Burkitt's lymphoma cell); ATCC CRL-2230, a strain of malignant lymphosarcoma cell from a 46 year old man with AIDS, which is positive for EB-virus and Kaposi sarcoma virus; EB-virus positive cell strain: ATCC CRL-1648(CA-46, a cell isolated from ascitic fluid of patient of American Burkitt's lymphoma).

Each strain is group into 2 test group. The group 1 is added with a preservation solution (10mMPB+0.2M NaCl phosphate buffer (pH7.4)) without the novel anti-tumor polypeptide. The group 2 is added with 200μg/ml the novel anti-tumor polypeptide 1 (plasmid pCHCEB11), the preservation solution is 10mMPB + 0.2M NaCl phosphate buffer, pH7.4.

After cultivation for 24 hours, the culture dish is added with the treating agents of the group described above. 72 hours after the addition of the treating agents, the culture dish is added with two types of fluorescent dyes, i.e. 20 μl of 50 μMol FITC and 20 μl of 50 uMol Rodamin-123, and observed under microscope Olympus IX-71 10 minutes later.

The result shows that the strain of EBV negative tumor cell grows well after the treatment of the novel anti-tumor polypeptide 1, and the most cells from every strain of EBV positive tumor cells appear the disappearance of mitochondrion and nucleus, is swelling and necrosis, most of them are dead. Apparently, compared with the PI stain experiment of Example 4, the result from the experiment of multi-fluorescence staining shows more clearly the powerful killing effect of the novel anti-tumor polypeptide 1 against EB virus positive tumor cell, see figure 6.

EBV negative tumor cells grow well, which means that the novel anti-tumor polypeptide does not attack the cell without surface antigen of EB virus in cell membrane. It is suggested that the novel anti-tumor polypeptide of the present invention has an ideal targeting specificity and safety.

Example 6. Killing effect of the novel anti-tumor polypeptide to solid tumor grown in naked mice

planted with cells of Burkitt's lymphoma caused by EB virus.

SCID immunodeficient mice are purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The mice are fed follow the standard feeding requirements. Water, bedding straw and feedstuff are all sterilized by high temperature or UV light. The mice are fed one weed under relative aseptic condition, and used in the inoculation experiment if there's no abnormality.

Cell suspensions of Raji (ATCC CCL-86) and 1648 (ATCC CRL-1648) in exponential phase are collected in 50 ml centrifuge tubes, centrifuged at 4 °C. The supernatant is then discarded. The cells are resuspended in 1640 liquid culture medium (plus calf serum) to 1.0×10^7 cells/ml. The mice are injected subcutaneously with 0.1 ml of cell suspension of Raji at left axillary, and with 0.1 ml of cell suspension of 1648(ATCC CRL-1648)at right axillary.

3-4 days after injection, the tumor grows into about 2 x 2 mm. The mice bearing the tumor are group into:

(group A) the preservation solution (10mM PBS+0.2M NaCl phosphate buffer(pH7.4)) without the anti-tumor polypeptide, as control group;

(group B) the novel anti-tumor polypeptide 1 (plasmid pCHCEB11), as treating group, 300 μg/mouse/day (calculated as 25 g), for 20 days continuously.

Ten mice of each group are injected subcutaneously 0.5 ml twice a day for 20 days continuously. The behavior of mice is observed and documented every day. The size of tumor is determined and photographed every two days.

The result (see figure 7) shows that the growth of tumor in group B of the novel anti-tumor polypeptide is inhibited significantly, wherein tumors in 7 mice disappear, and tumors in the other 3 mice are smaller clearly than that of control group. The novel anti-tumor polypeptide is effective to inhibit the growth of solid tumor in mice caused by EBV positive cells of lymphosarcoma. But the novel anti-tumor polypeptide is ineffective to inhibit the growth of solid tumor in mice caused by EBV negative cells of lymphosarcoma.

Example 7. Pathological observation of in vivo experiment of tumor elimination

Histopathology observation of tumors: Mice are sacrificed at the end of the experiment of Example 6. Tumors are extracted, fixed in 10% formalin. The paraffin slices are HE stained and observed under routine optical microscopy.

Observed under the microscopy, the solid tumors from mice of control group is proliferating

vigorously; the cells of EBV positive solid tumors from mice of group of the novel anti-tumor polypeptide shrink remarkably. Most of the cell masses in the section are necrotic tumor cells, and a large amount of peritumoral lymphocytic infiltration is observed. The histopathology result suggests that during the treatment of 20 days, the novel anti-tumor polypeptide killed nearly all of the tumor cells in the solid tumor (see figure 8, D).

CLAIMS

1. A polypeptide for treating tumors caused by EB virus, said polypeptide being formed by operable linkage of a mutant polypeptide of colicin which can form ion channels with a polypeptide of anti-EB virus antibody or a polypeptide of anti-EB virus antibody mimetics, the mutant polypeptide of colicin which can form ion channels is obtained by mutation of amino acid residues of G11A, H22G, A26G, V31L, and H40D to peptide chain of wild-type colicin 1a, wherein the polypeptide of anti-EB virus antibody is the monoclonal antibody secreted by hybridoma of ATCC HB-168 and the polypeptide of antibody mimetics comprises: CDR1 heavy chain of anti-EB virus antibody; CDR1-CDR2 heavy chain linking segment of anti-EB virus antibody; and CDR3 light chain of anti-EB virus antibody.

2. The polypeptide for treating tumors caused by EB virus according to claim 1, wherein the polypeptide against tumor caused by EB virus has the amino acid sequence shown in SEQ ID NO. 29.

3. A nucleic acid encoding the polypeptide for treating tumors caused by EB virus of claim 1 or 2.

4. The nucleic acid according to claim 3, which has the nucleotide sequence shown in SEQ ID NO. 30.

5. A recombination plasmid comprising the nucleic acid of claim 3.

6. A preparation method for the polypeptide for treating tumors caused by EB virus of claim 1 or 2, comprising steps of: transforming the recombination plasmid of claim 5 into an expression system for expression, and isolating the polypeptide expressed.

7. Use of the polypeptide for treating tumors caused by EB virus of claim 1 or 2 in preparation of a medicament for the treatment and prevention of tumor caused by EB virus.

8. A mutant colicin 1a polypeptide, its amino acid sequence is shown in SEQ ID NO. 24.

9. A nucleic acid encoding the mutant colicin 1a polypeptide of claim 8.

10. Use of the polypeptide prepared according to the method of claim 6 for treating tumors caused by EB virus.

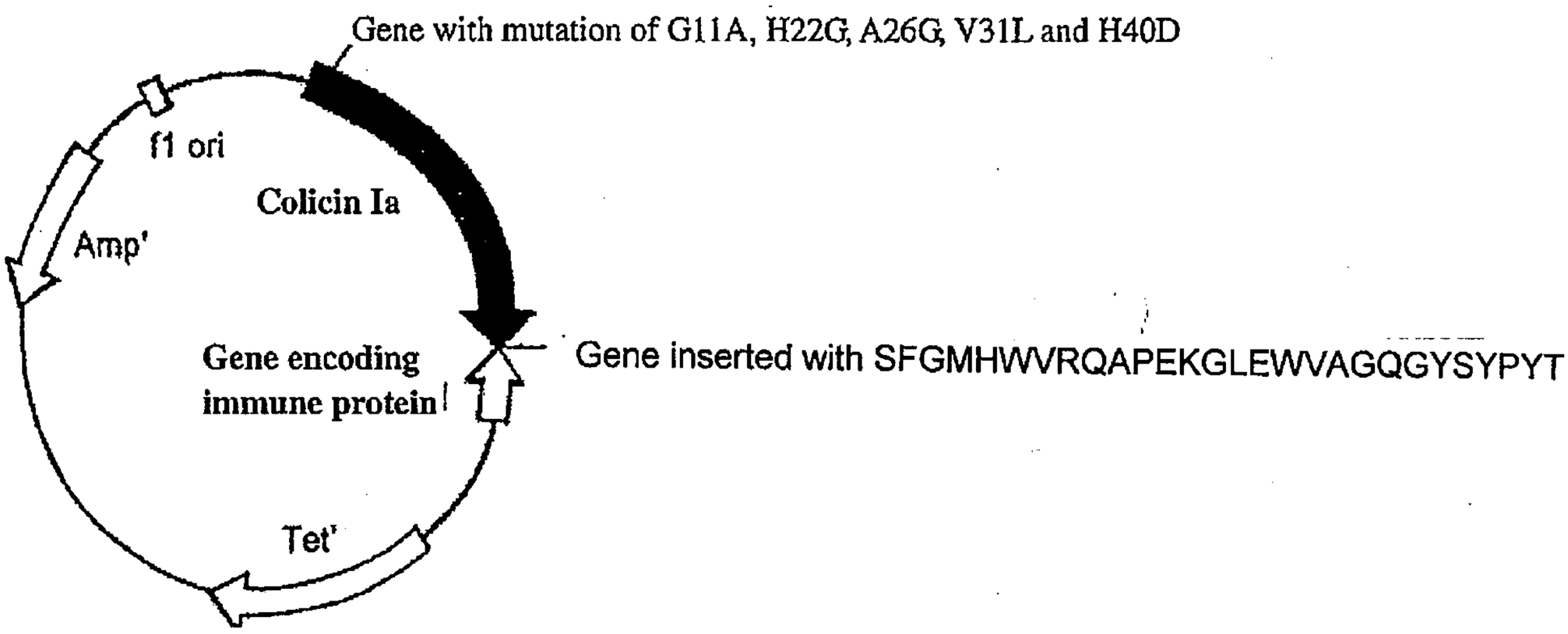


Figure 1

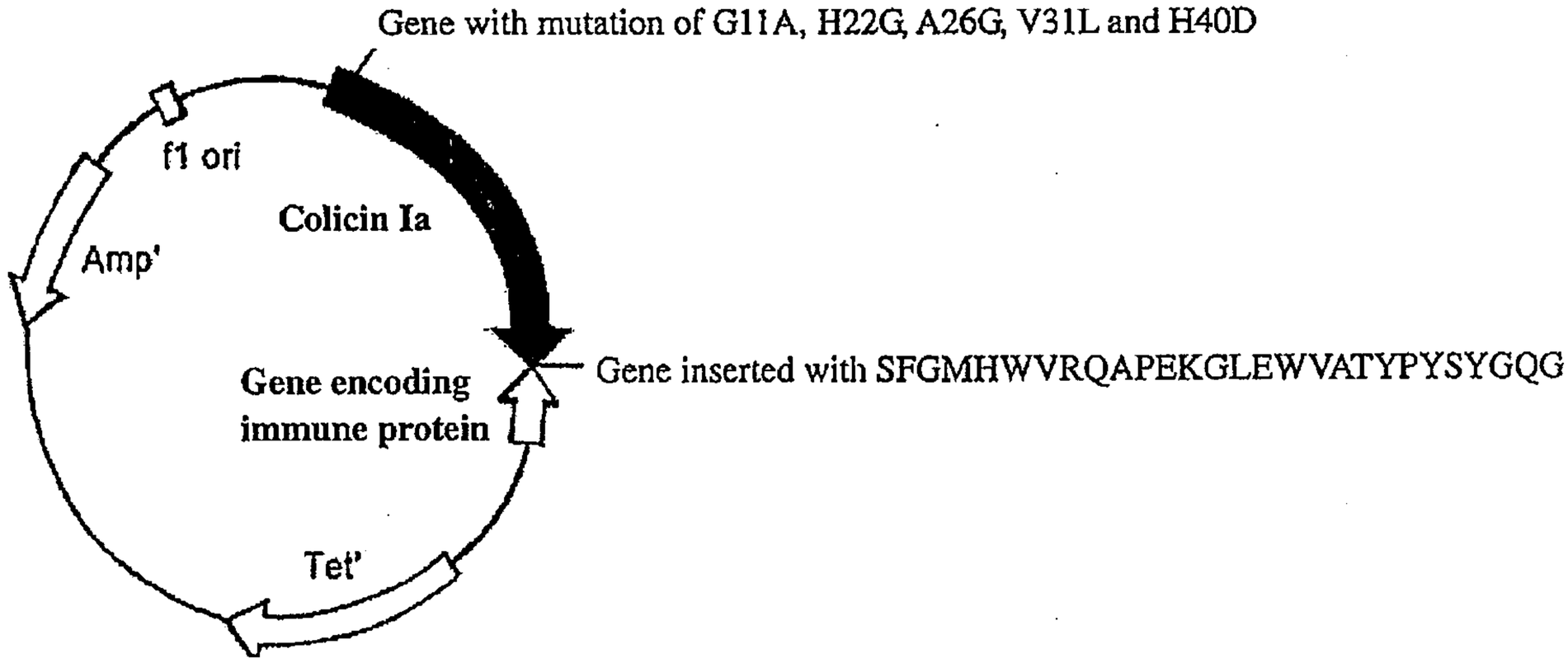


Figure 2

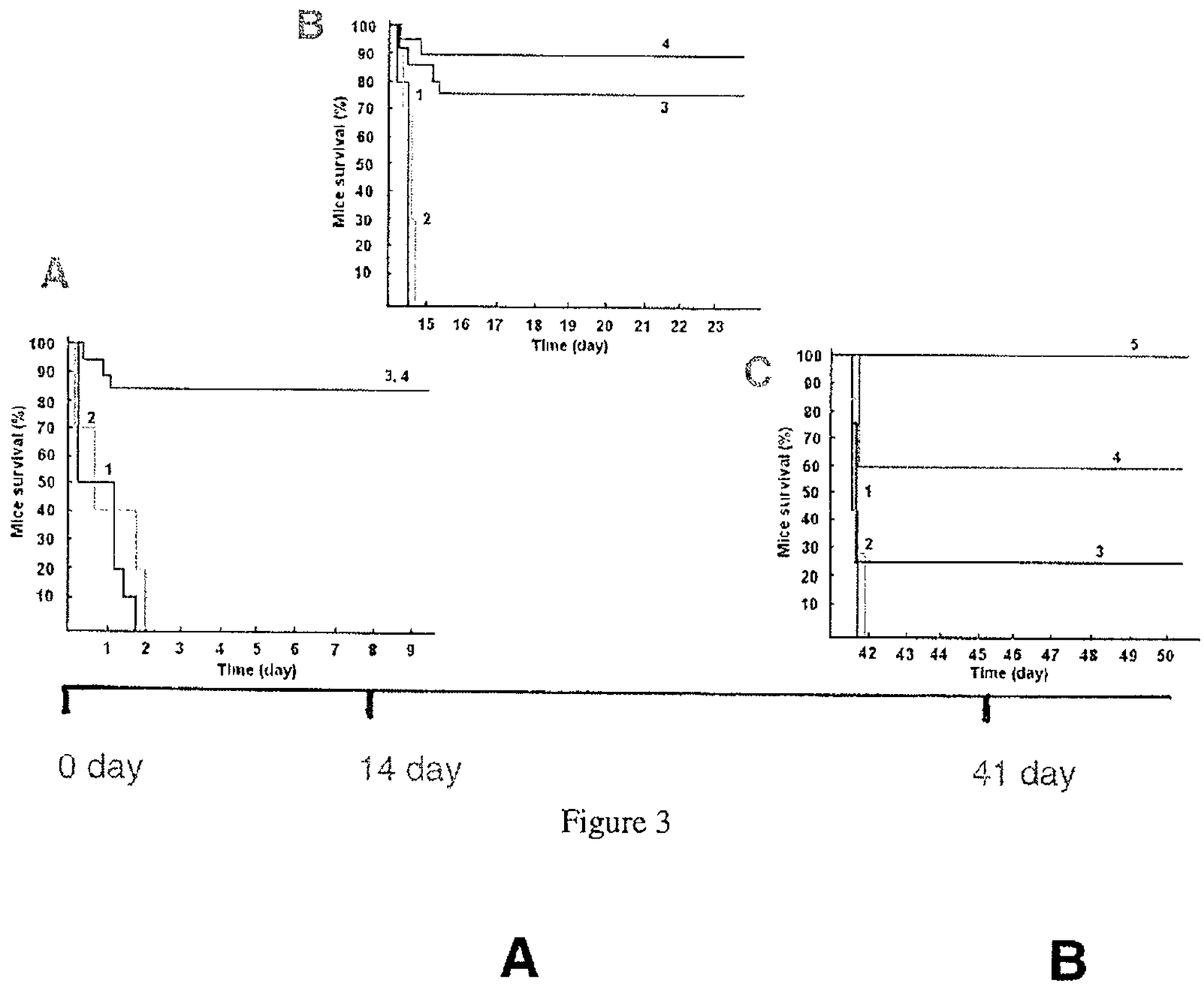


Figure 3

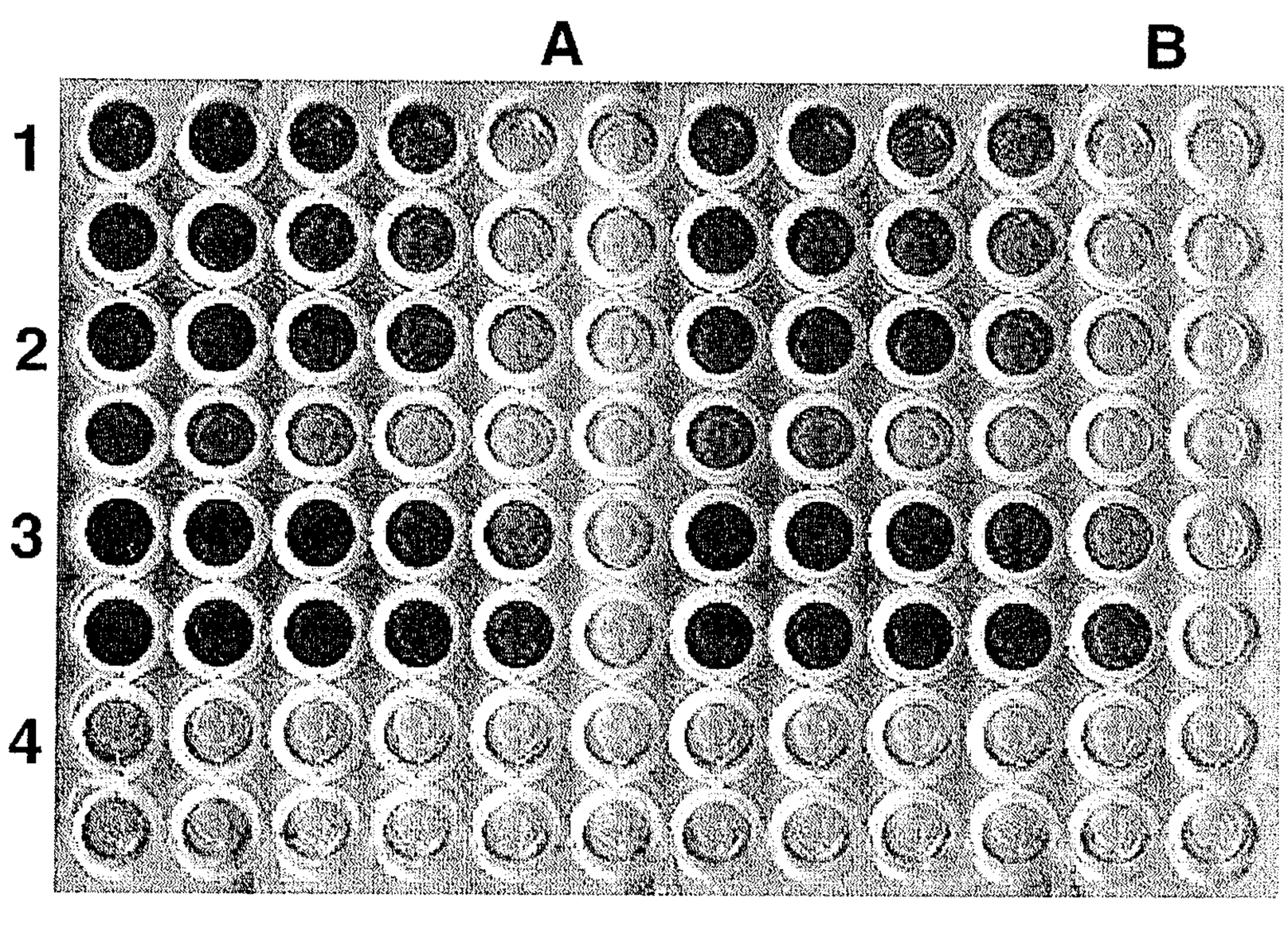


Figure 4

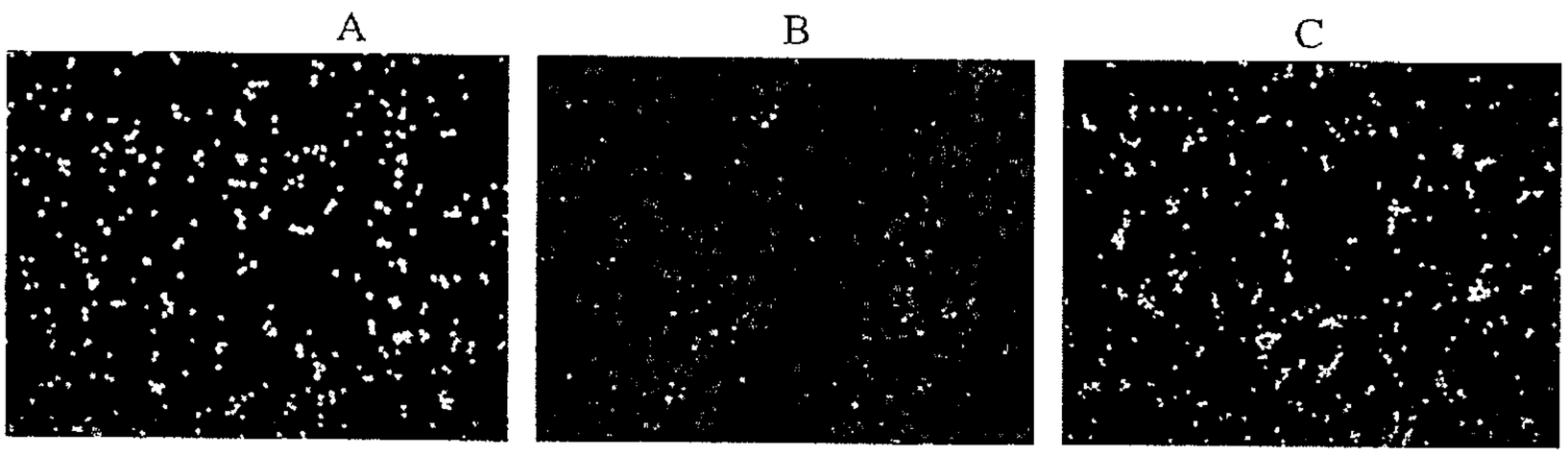


Figure 5

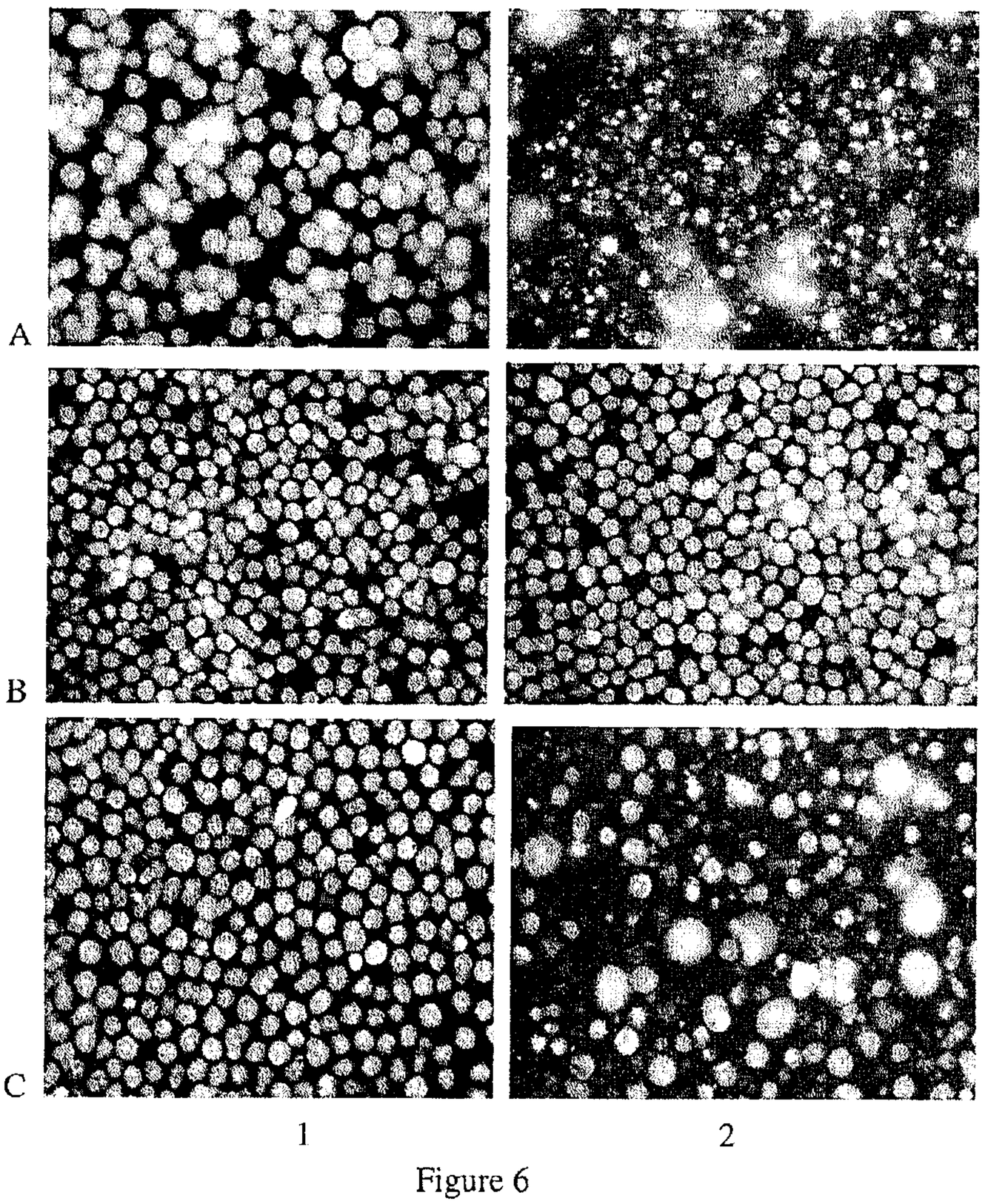


Figure 6

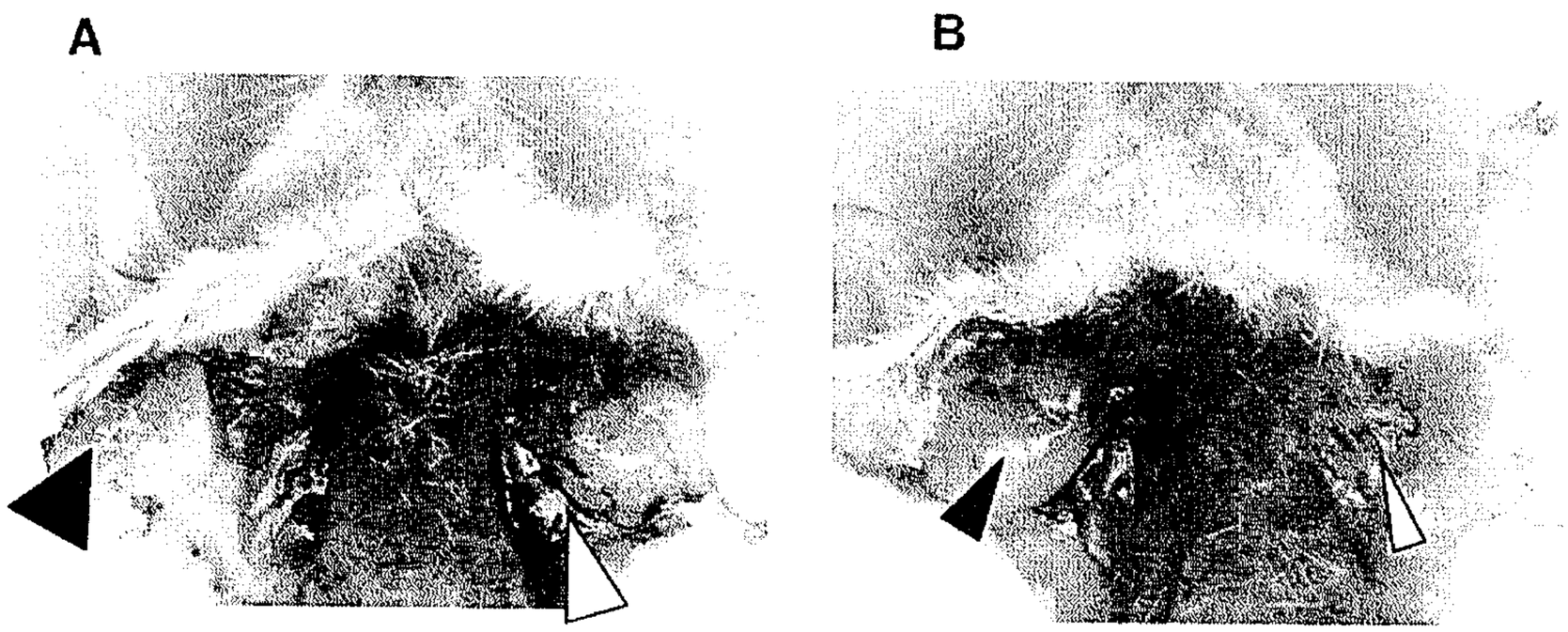


Figure 7

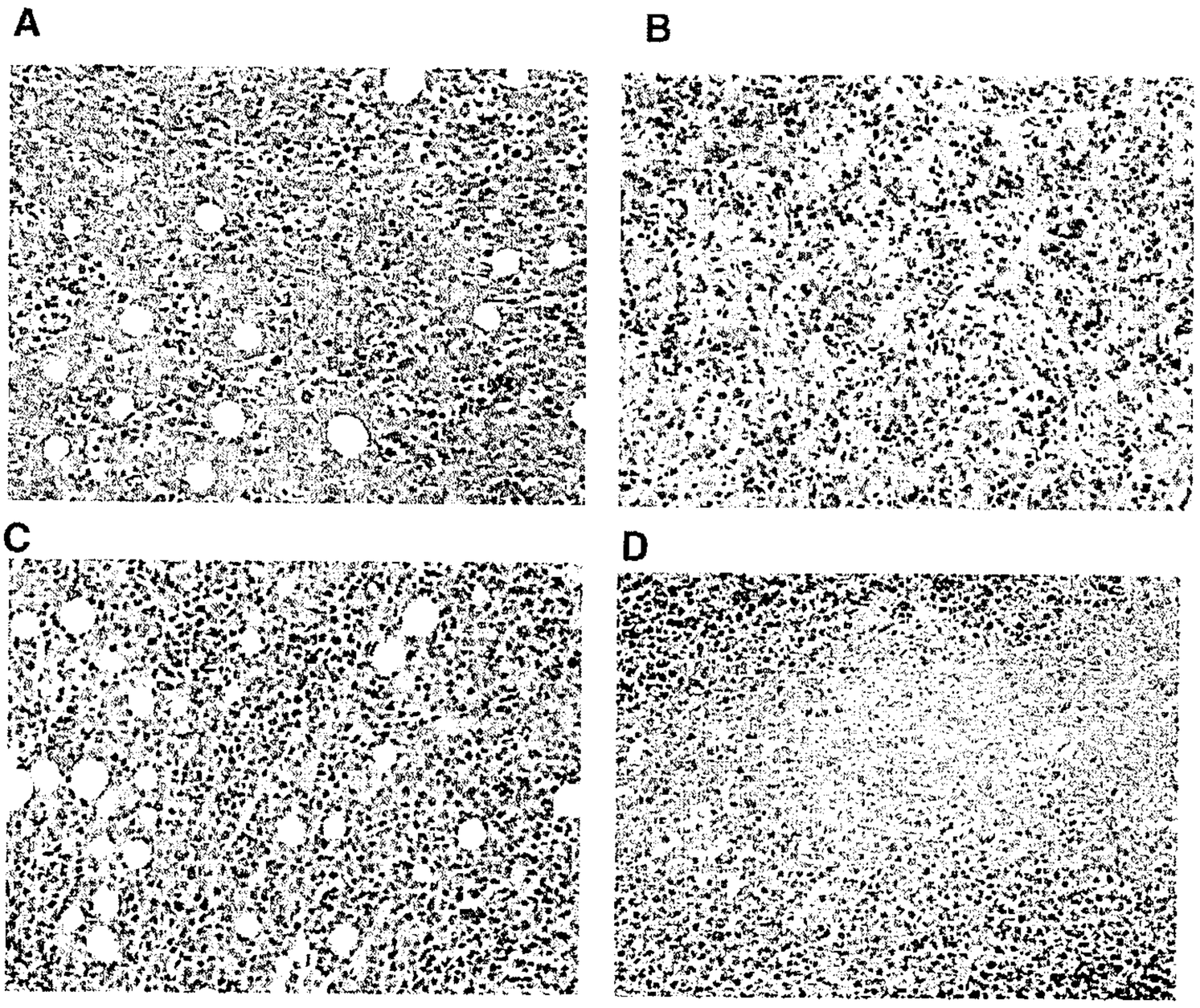


Figure 8