Diethylenetriamine-N,N,N',N",N"-pentaacetic acid) and added to each well, and allowed to react overnight at 4°C. After washing three times with 300 μl of washing solution, 200 μl of buffer C containing 10 ng of Eu-Labeled Streptavidin (available from PERKIN ELMER) and 25 ng of biotin-labeled PcrV was added, and allowed to react for 1 hour at room temperature. After the reaction, washing three times with 300 μl of washing solution, and 200 μl of enhancemnt reagent (1.39 g/l potassium phthalate, 19.3 mg/l of Tri-n-octylphosphine oxide, 4.59 mg/l of 2-naphthoyltrifluoroacetone, 1.0 g/l of Triton-X100, 6.0 g/l of acetic acid) was added, and time-resolved fluorescence was measured.

[0053]

From the result of screening, 20 clones of hybridoma which exhibited strong affinity with recombinant PcrV were selected, and cytotoxicity inhibition activity by Pseudomonas aeruginosa was examined according to Reference ~~E~~example ~~4~~5. As a result, cytotoxicity inhibiting activity was observed in 10 clones, and these clones were then cloned twice by limiting dilution method, and thus hybridoma cells were selected. From the obtained 10 clones, 4 clones exhibited high cytotoxicity inhibition activity were selected, and named 1F3, 2A4, 6F5, and 7A7, respectively. For these antibodies, subclass of antibody was determined using mouse monoclonal antibody isotyping ELISA kit (available from BD Biosciences), and it was found that 1F3 was IgG2a, 2A4 was IgG2b, 6F5 was IgG2a, 7A7 was IgG2a.

[0054]

Hybridomas cells that produce monoclonal antibodies 1F3 and 2A4 were deposited with National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Center No.6, 1-1-1, Higashi, Tsukuba-shi, IBARAGI, JAPAN) on January 15, 2009, under the accession numbers of FERM ABP-11805 and FERM ABP-11806, respectively.

Reference example 3

[0055]

Binding activity of antibody

For measuring binding activity of antibodies (1F3, 2A4, 6F5, 7A7), competitive immunoassay was performed. Each well of a 96-well microtiter plate (available from Nunc) was added with 100 μl of tris buffer (50 mM Tris-HCl, pH7.5) containing 1.5 μg of anti-mouse Fc antibody (available from Jackson ImmunoReseach)

and immobilized for 16 hours at 4°C. These wells were washed twice with 300 μl of washing solution, then added with 300 μl of BlockAce (available from Dainippon Sumitomo Pharma Co., Ltd.) including 10% sucrose and left for two hours at room temperature to achieve blocking (anti-mouse IgG antibody solid-phased plate). After washing twice with 300 μl of washing solution, 2 ng/well of each antibody and non-labeled PcrV at five concentrations in 10-fold dilution series from 500 ng/well were added. Then, 5 ng/well of biotinylated PcrV was added and allowed to react overnight. After washing four times with 300 μl of washing liquid and adding with 100 μl/well of Enhancement Reagent (available from Wallac), time-resolved fluorescence was measured after stirring for 1 minute. As a result, 1F3, 2A4, 6F5 and 7A7 showed stronger binding activity against PcrV than Mab166 (Fig. 1).

[0056]

Next, affinity of 1F3, 2A4, 6F5, 7A7 and Mab166 with PcrV was determined by surface plasmon resonance analysis. Anti-mouse antibody was immobilized on a CM5 sensor chip by using Mouse Antibody Capture Kit (available from BIACORE). Sequentially, each PcrV antibody was captured. The recombinant PcrV was loaded on BIAcore T100 instrument with the sensor chip to determine the affinity.

[0057]

As a result, every clone showed higher affinity than Mab166 as evidenced from the affinity of $3.7 \times 10^{-10}$ for 1F3, the affinity of $3.5 \times 10^{-10}$ for 2A4, the affinity of $1.1 \times 10^{-10}$ for 6F5, and the affinity of $1.1 \times 10^{-9}$ for 7A7, in contrast to the affinity of $3.0 \times 10^{-9}$ for Mab166 (Fig. 2).

Reference example 4

[0058]

Sandwich immunoassay with Mab166

In order to prove that 1F3, 2A4, 6F5 and 7A7 have a different epitope from that of Mab166, sandwich immunoassay between each antibody and Mab166 was examined.

First, Mab166 was labeled with biotin. One hundred μg of Mab166 and 7.853 μg of NHS-PEO4 Biotin (available from PIERCE) were mixed in 0.1M PBS (pH 7.4), and allowed to react for 2 hours on ice. Thereafter, gel chromatography (G2000SW column (available from TOSOH)) was carried out to remove unreacted biotin from the reaction solution.

membrane. The transferred membrane was blocked with Block Ace (available from Dainippon Sumitomo Pharma Co., Ltd.) at room temperature for 2 hours under shaking. PcrV antibody solution diluted into 1 μg/mL was added to the membrane, allowed to react overnight at 4°C, and then washed with washing buffer B (10 mM phosphate buffer (pH 7.4), 0.05% Tween 20). As secondary antibody, labeled anti-mouse IgG antibody (available from GE Healthcare) solution was added to the membrane, and allowed to react for 2 hours at room temperature. Thereafter, the membrane was washed with washing buffer B, and the signal was detected by ECL Plus Western Blot Detection System (available from GE Healthcare) and visualized by LAS-1000 (available from FUJIFILM) (Fig. 7). While PcrV neutralization antibodies 1F3 and 2A4 reacted with both of full-length PcrV and truncated PcrV, m166 as well as ~~6F5~~9D12 and ~~7A7~~12H9 reacted only with full-length PcrV, and did not react with truncated PcrV.

[0066]

This demonstrated that epitope region of PcrV neutralization antibodies 1F3 and 2A4 was a region corresponding to amino acid residues 136-233, and m166, ~~6F5~~ 9D12 and ~~7A7~~12H9 did not exclusively have an epitope region corresponding to amino acid residues 136-233.

Reference example 7

[0067]

Correlation between specific region of PcrV protein and strength of cytotoxicity

Using full-length PcrV protein (SEQ ID NO: 1) and truncated PcrV protein (having amino acid sequence corresponding to positions 136 to 233 in SEQ ID NO:1), suppression test of cytotoxicity inhibiting activity in 1F3, 2A4 and m166 were conducted in the following manner.

First, 1F3, 2A4 and m166 were diluted by serial doubling dilution starting with 200nM, 200nM and 400nM, respectively, and 10 μL of these antibodies were added to the 96-well plate. In this test, the test consentration range of 1F3, 2A4 and m166 were adjusted to 1.56-6.25 nM, 6.25-25 nM and 50-200 nM, respectively, For each test concentration range, each 10 μl of full-length PcrV protein or truncated PcrV protein in molar ratios of 30, 10, 3, 1 and 0.3 folds was added to 96-well plate, and kept still for 30 minutes at room temperature. Next, myeloma cell P3U1 was prepared

Claims

[1] A humanized monoclonal antibody against PcrV or a part thereof, having at least one feature selected from (A) to (D):

(1) inhibiting 50% or more of cytotoxicity to leukocyte cell of Pseudomonas aeruginosa at a concentration of 1 nM to 200 nM in vitro;

(2) inhibiting 50% or more of cytotoxicity to myeloma cell of Pseudomonas aeruginosa at a concentration of 1 nM to 50 nM in vitro;

(3) having a dissociation constant (Kd) with PcrV of $2 \times 10^{-9}$ (M) or less; and

(4) having its epitope at positions of 136 to 233 in amino acid sequence of SEQ ID NO: 1;

[2] A humanized monoclonal antibody or a part thereof, having amino acid sequence where complementarity determining region (CDR) of monoclonal antibody produced by hybridoma deposited as an accession number of FERM ABP-11805.

[3] A humanized monoclonal antibody against PcrV or a part thereof, having

1) in a complementarity determining region, a heavy chain variable region including the following amino acid sequence: SFTSYWMH (SEQ ID NO: 15), INPSNGRTNYNEKFNT (SEQ ID NO: 16), YGNYVVYYTMDY (SEQ ID NO: 17) and

2) in a complementarity determining region, a light chain variable region including the following amino acid sequence: SASTSVSYME (SEQ ID NO: 18), TTSKLAS (SEQ ID NO: 19), HQWRNYPFT (SEQ ID NO: 20).

[4] A humanized monoclonal antibody against PcrV or a part thereof, having

1) in a complementarity determining region, a heavy chain variable region including the following amino acid sequence: SFTSYWMH (SEQ ID NO: 15), INPSNGRTNYNEKFNT (SEQ ID NO: 16), YGNYVVYYTMDY (SEQ ID NO: 17) or in at least one CDR of the three CDR set, a heavy chain variable region including those having substitution, addition or deletion of one or several amino acid and

2) in a complementarity determining region, a light chain variable region including the following amino acid sequence: SASTSVSYME (SEQ ID NO: 18), TTSKLAS (SEQ ID NO: 19), HQWRNYPFT (SEQ ID NO: 20) or in at least one CDR of the three CDR set, a light chain variable region including those having substitution, addition or deletion of one or several amino acid,

and at least one feature selected from (1) to (4):

(1) inhibiting 50% or more of cytotoxicity to leukocyte cell of Pseudomonas

aeruginosa at a concentration of 1 nM to 200 nM in vitro;

(2) inhibiting 50% or more of cytotoxicity to myeloma cell of Pseudomonas aeruginosa at a concentration of 1 nM to 50 nM in vitro;

(3) having a dissociation constant (Kd) with PcrV of $2 \times 10^{-9}$ (M) or less; and

(4) having its epitope at positions of 136 to 233 in amino acid sequence of SEQ ID NO: 1.

[5] A humanized monoclonal antibody against PcrV or a part thereof, having 1) a heavy chain variable region having amino acid sequence of SEQ ID NO: 27, and 2) a light chain variable region having amino acid sequence of SEQ ID NO: 28.

[6] A pharmaceutical composition comprising the antibody or a part thereof according to any one of claim 1 to 5, as an active ingredient.

[7] A polynucleotide encoding a heavy chain variable region and a light chain variable region of antibody according to any one of claim 3 to 5.

[8] An expression vector comprising of the polynucleotide according to claim 7.

## Claims

[1] A humanized monoclonal antibody against PcrV or a region which is a part of the antibody and binds specifically to PcrV, having at least one feature selected from (1) to (2):

(1) inhibiting 50% or more of cytotoxicity to leukocyte cell of Pseudomonas aeruginosa, U937, at a concentration of 1 nM to 25 nM in vitro; and

(2) inhibiting 50% or more of cytotoxicity to myeloma cell of Pseudomonas aeruginosa, U3P1, at a concentration of 1 nM to 20 nM in vitro.

[2] A humanized monoclonal antibody or a region which is a part of the antibody and binds specifically to PcrV, comprising amino acid sequences of all complementarity determining regions (CDR1 to 3) of monoclonal antibody produced by hybridoma deposited as an accession number of FERM BP-11085, as amino acid sequences of the identical CDR1 to 3.

[3] A humanized monoclonal antibody against PcrV or a region which is a part of the antibody and binds specifically to PcrV, comprising

1) in a complementarity determining region, a heavy chain variable region including the following amino acid sequence: SFTSYWMH (SEQ ID NO: 15) as CDR1, INPSNGRTNYNEKFNT (SEQ ID NO: 16) as CDR2 and YGNYVVYYTMDY (SEQ ID NO: 17) as CDR3, and

2) in a complementarity determining region, a light chain variable region including the following amino acid sequence: SASTSVSYME (SEQ ID NO: 18) as CDR1, TTSKLAS (SEQ ID NO: 19) as CDR2 and HQWRNYPFT (SEQ ID NO: 20) as CDR3.

[4] A humanized monoclonal antibody against PcrV or a region which is a part of the antibody and binds specifically to PcrV, comprising

1) in a complementarity determining region, a heavy chain variable region including the following amino acid sequence: SFTSYWMH (SEQ ID NO: 15) as CDR1, INPSNGRTNYNEKFNT (SEQ ID NO: 16) as CDR2 and YGNYVVYYTMDY (SEQ ID NO: 17) as CDR3, and

2) in a complementarity determining region, a light chain variable region including the following amino acid sequence: SASTSVSYME (SEQ ID NO: 18) as CDR1, TTSKLAS (SEQ ID NO: 19) as CDR2 and HQWRNYPFT (SEQ ID NO: 20) as CDR3, and having at least one feature selected from (1) to (4):

(1) inhibiting 50% or more of cytotoxicity to leukocyte cell of Pseudomonas

aeruginosa, U937, at a concentration of 1 nM to 200 nM in vitro;

(2) inhibiting 50% or more of cytotoxicity to myeloma cell of Pseudomonas aeruginosa, U3P1, at a concentration of 1 nM to 50 nM in vitro;

(3) having a dissociation constant (Kd) with PcrV of $2 \times 10^{-9}$ (M) or less; and

(4) at positions of 136 to 233 in amino acid sequence of SEQ ID NO: 1 is the epitope recognized by the antibody or region.

[5] A humanized monoclonal antibody against PcrV or a region which is a part of the antibody and binds specifically to PcrV, comprising

1) a heavy chain variable region comprising amino acid sequence of SEQ ID NO: 27, and

2) a light chain variable region comprising amino acid sequence of SEQ ID NO: 28.

[6] A humanized monoclonal antibody against PcrV or a region which is a part of the antibody and binds specifically to PcrV, comprising

1) a heavy chain variable region comprising amino acid sequence with 95% or more similarity to that of SEQ ID NO: 27, and

2) a light chain variable region comprising amino acid sequence with 95% or more similarity to that of SEQ ID NO: 28,

and having at least one feature selected from (1) to (4):

(1) inhibiting 50% or more of cytotoxicity to leukocyte cell of Pseudomonas aeruginosa, U937, at a concentration of 1 nM to 200 nM in vitro;

(2) inhibiting 50% or more of cytotoxicity to myeloma cell of Pseudomonas aeruginosa, U3P1, at a concentration of 1 nM to 50 nM in vitro;

(3) having a dissociation constant (Kd) with PcrV of $2 \times 10^{-9}$ (M) or less; and

(4) at positions of 136 to 233 in amino acid sequence of SEQ ID NO: 1 is the epitope recognized by the antibody or region.

[7] A pharmaceutical composition comprising the antibody or the region which is a part of the antibody and binds specifically to PcrV, according to any one of claims 1 to 6, as an active ingredient.

[8] A polynucleotide encoding a heavy chain variable region or a light chain variable region of the antibody according to any one of claims 1 to 6.

[9] An expression vector comprising of the polynucleotide according to claim 8.