[Designation of Document] SPECIFICATION

[Title of the Invention] NOVEL ANTI-DR5 ANTIBODY

[Technical Field]

The present invention relates to an antibody which binds to a cell surface receptor involved in apoptosis induction and is useful as a therapeutic and/or preventive agent for tumors, and also relates to a method of treating and/or preventing cancer, an autoimmune disease, or an inflammatory disease using the antibody.

[Background Art]

Apoptosis is a phenomenon essential to the physiological process of removing unnecessary cells or damaged cells and maintaining the number of normal cells in vivo. Owing to progress in elucidation of the fact that the regulatory mechanism of apoptosis is often impaired in cancer or immune diseases and also elucidation of the regulatory pathway of apoptosis, the development of a novel apoptosis inducer which can be used in the treatment of cancer or immune diseases has been advanced. In particular, an antibody which has a binding affinity for a ligand for a cell surface receptor involved in apoptosis induction typified by a death receptor or an antibody which has a binding affinity for said death receptor is expected to have a therapeutic effect on these diseases (see, for example, Non-patent document 1). Death receptor 5 (DR5) which is one of the death receptors is sometimes also called KILLER, TRICK

2A, TRAIL-R2, TRICK B, or CD262, and a plurality of agonistic antibodies which induce apoptosis in cells are known (see, for example, Non-patent document 2 or 3, or Patent documents 1 to 6). Some antibodies are currently being developed in clinical trials as candidate therapeutic agents and are expected to have a therapeutic effect such that the antibodies specifically act in an agonistic manner on cells (cancer cells or immune disease-related cells) which express the receptor in order to kill the cells. In order for such an antibody to have an antitumor effect, it is essential that the cells express DR5, however, it has been revealed that there is no correlation between the effect and the expression level of DR5 in a preclinical trial (Non-patent document 4). It is considered that this is because a cellular response is regulated by many factors such as the expression level of intracellular signaling molecules (such as caspase-8 or Bcl-2) involved in apoptosis pathways (Non-patent document 5).

[Prior Art Document]

[Patent document 1]

WO 98/51793

[Patent document 2]

WO 2001/83560

[Patent document 3]

WO 2002/94880

[Patent document 4]

WO 2003/54216

[Patent document 5]

WO 2006/83971

[Patent document 6]

WO 2007/22157

[Non-patent document 1]

Cell Death and Differentiation, 10: 66-75 (2003)

[Non-patent document 2]

Journal of Immunology, 162: 2597-2605 (1999)

[Non-patent document 3]

Nature Medicine, 7(8): 954-960 (2001)

[Non-patent document 4]

Cell Death and Differentiation, 10: 66-75 (2003)

[Non-patent document 5]

Journal of Clinical Oncology, 26: 3621-3630 (2008)

[Summary of the Invention]

[Problems that the Invention is to Solve]

An object of the invention is to provide an antibody or a functional fragment of the antibody to be used in a pharmaceutical having a therapeutic effect on cancer, and a polynucleotide encoding the antibody or a functional fragment of the antibody.

[Means for Solving the Problems]

The present inventors made intensive studies in order to achieve the above object, and as a result, they found an

antibody which exhibits a potent apoptosis-inducing activity in cells, and thus completed the invention. This also brings about an effective therapeutic effect in patients in whom a sufficient therapeutic effect of currently available antibodies cannot be obtained.

That is, the invention includes the following inventions.

(1) An antibody characterized in that:

a heavy chain sequence contains a variable region having CDRH1, CDRH2, and CDRH3, and the CDRH1 comprises an amino acid sequence represented by SEQ ID NO: 82, the CDRH2 comprises either one of the amino acid sequences represented by SEQ ID NOS: 83 and 89, and the CDRH3 comprises an amino acid sequence represented by SEQ ID NO: 84; and

a light chain sequence contains a variable region having CDRL1, CDRL2, and CDRL3, and the CDRL1 comprises any one of the amino acid sequences represented by SEQ ID NOS: 79, 85, 86, 87, and 88, the CDRL2 comprises an amino acid sequence represented by SEQ ID NO: 80, and the CDRL3 comprises an amino acid sequence represented by SEQ ID NO: 81, or a functional fragment of the antibody.

(2) The antibody or a functional fragment of the antibody according to (1), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 20

and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 16.

(3) The antibody or a functional fragment of the antibody according to (1) or (2), characterized in that the antibody is a chimeric antibody.

(4) The antibody or a functional fragment of the antibody according to (3), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 20 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 16.

(5) The antibody or a functional fragment of the antibody according to (1), characterized in that the antibody is humanized.

(6) The antibody or a functional fragment of the antibody according to (5), characterized by containing:

(a) a heavy chain variable region sequence selected from the group consisting of the following amino acid sequences:

a1) an amino acid sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 42;

a2) an amino acid sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70;

a3) an amino acid sequence having a homology of at least 95% with an amino acid sequence selected from a1) and a2);

a4) an amino acid sequence having a homology of at least 99% with an amino acid sequence selected from a1) and a2); and

a5) an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in either one of the amino acid sequences selected from a1) and a2); and

(b) a light chain variable region sequence selected from the group consisting of the following amino acid sequences:

b1) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 28;

b2) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 52;

b3) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 58;

b4) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 62;

b5) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 66;

b6) an amino acid sequence having a homology of at least 95% with any one of the amino acid sequences selected from b1) to b5);

b7) an amino acid sequence having a homology of at least 99% with any one of the amino acid sequences selected from b1) to b5); and

b8) an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in any one of the amino acid sequences selected from b1) to b5).

(7) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 42 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 28.

(8) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 52.

(9) The antibody or a functional fragment of the antibody

according to (6), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 58.

(10) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 62.

(11) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 66.

(12) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 42 and a light chain sequence comprising amino acid residues 21 to 239 of the

amino acid sequence represented by SEQ ID NO: 28.

(13) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 52.

(14) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 58.

(15) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 62.

(16) The antibody or a functional fragment of the antibody according to (6), characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 66.

(17) The functional fragment of the antibody according to any one of (1) to (16), which is selected from the group consisting of Fab, F(ab')2, Fab' and Fv.

(18) A pharmaceutical composition characterized by comprising at least one of the antibodies or functional fragments of the antibodies according to (1) to (17).

(19) The pharmaceutical composition according to (18), characterized by being a pharmaceutical composition for treating and/or preventing cancer.

(20) A pharmaceutical composition for treating and/or preventing cancer, characterized by comprising at least one of the antibodies or functional fragments of the antibodies according to (1) to (17) and at least one member selected from the group consisting of paclitaxel, carboplatin, CPT-11, and vinblastine.

(21) The pharmaceutical composition according to (19) or (20), wherein the cancer is selected from the group consisting of lung cancer, prostate cancer, thyroid cancer, stomach cancer, liver cancer, ovarian cancer, colon cancer, breast cancer, pancreatic cancer, renal cancer, uterine cancer, melanoma, fibrosarcoma, glioblastoma, and blood cell cancer.

(22) A method of treating and/or preventing cancer, characterized by administering at least one of the antibodies or functional fragments of the antibodies according to (1) to (17).

(23) A method of treating and/or preventing cancer, characterized by simultaneously or sequentially administering at least one of the antibodies or functional fragments of the antibodies according to (1) to (17) and at least one member selected from the group consisting of paclitaxel, carboplatin, CPT-11, vinblastine, and 5-FU.

(24) The treatment and/or prevention method according to (22) or (23), wherein the cancer is selected from the group consisting of lung cancer, prostate cancer, thyroid cancer, stomach cancer, liver cancer, ovarian cancer, colon cancer, breast cancer, pancreatic cancer, uterine cancer, melanoma, glioblastoma, and blood cell cancer.

(25) A polynucleotide encoding the antibody according to any one of (2), (4), and (6) to (16).

(26) The polynucleotide according to (25), characterized by containing a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 19 and a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 15.

(27) The polynucleotide according to (25), characterized by containing a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 19 and a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 15.

(28) The polynucleotide according to (25), characterized

by containing:

(a) a polynucleotide selected from the group consisting of the following nucleotide sequences:

a1) a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 41;

a2) a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69;

a3) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to a nucleotide sequence selected from a1) and a2) under stringent conditions; and

a4) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in a nucleotide sequence selected from a1) and a2); and

(b) a polynucleotide selected from the group consisting of the following nucleotide sequences:

b1) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 27;

b2) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 51;

b3) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 57;

b4) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 61;

b5) a nucleotide sequence comprising nucleotides 61 to

402 of the nucleotide sequence represented by SEQ ID NO: 65;

b6) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from b1) to a5) under stringent conditions; and

b7) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in any one of the nucleotide sequences selected from b1) to b5).

(29) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 41, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 27.

(30) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 51.

(31) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to

402 of the nucleotide sequence represented by SEQ ID NO: 57.

(32) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 61.

(33) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 65.

(34) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 41, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 27.

(35) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 51.

(36) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 57.

(37) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 61.

(38) The polynucleotide according to (28), characterized by containing a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 65.

(39) A vector comprising any one of the polynucleotides according to (25) to (38).

(40) A transformed host cell comprising any one of the polynucleotides according to (25) to (38).

(41) A transformed host cell comprising the vector according to (39).

(42) A method of producing the antibody according to any

one of (2), (4), and (6) to (16), comprising the step of culturing the host cell according to (40) or (41) and purifying the antibody from the resulting cultured product.

(43) An antibody or a functional fragment of the antibody, characterized by binding to the same epitope as an antibody containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 20 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 16.

(44) An antibody or a functional fragment of the antibody, characterized by competing with an antibody containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 20 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 16.

(45) The antibody or a functional fragment of the antibody according to (43) or (44), characterized in that a Fab fragment of the antibody prepared by papain digestion, when binding the recombinant protein represented by SEQ ID NO: 23, lies adjacent to the glycine residue at position 26, the isoleucine residue at position 34, the glutamic acid residue at position 36, the aspartic acid residue at position 37, the glycine residue at position 38, the aspartic acid residue at position 56, the leucine residue at position 57, the leucine

residue at position 58, the phenylalanine residue at position 59, the leucine residue at position 61, and the arginine residue at position 62 of the recombinant protein represented by SEQ ID NO: 23 at a distance of 4 Å or less.

(46) The antibody or a functional fragment of the antibody according to (45), characterized in that the distance between each amino acid residue constituting the recombinant protein represented by SEQ ID NO: 23 and the Fab fragment is determined by a complex structural analysis using X-ray diffraction data.

[Advantage of the Invention]

According to the invention, a therapeutic agent for cancer whose mechanism of action is primarily through apoptosis induction in cells can be obtained.

[Brief Description of the Drawings]

[Fig. 1]

Fig. 1 is a figure showing the cytocidal effect of a mouse B273 antibody.

[Fig. 2]

Fig. 2 is a figure showing binding activities of a cB273 antibody and sTRAIL to a DR5 extracellular domain protein.

[Fig. 3]

Fig. 3 is a figure showing the binding activity of a cB273 antibody to human DR5 using Biacore. On the upper side of the figure, a measurement chart is shown, in which the ordinate

represents resonance units (RU), and the abscissa represents time (sec). On the lower side of the figure, Kon, Koff, and KD values of the cB273 antibody calculated using analysis software are shown.

[Fig. 4]

Fig. 4 is a figure showing the in vitro cytocidal effect of a cB273 antibody on human cancer cell lines. A) shows the results for a human ovarian cancer cell line, B) shows the results for a human colon cancer cell line, C) shows the results for a human lung cancer cell line, and D) shows the results for a human breast cancer cell line.

[Fig. 5]

Fig. 5 is a figure showing the in vitro cytocidal effect of a cB273 antibody on human cancer cell lines. A) shows the results for a human pancreatic cancer cell line, B) shows the results for a human melanoma cell line, C) shows the results for a human glioblastoma cell line, and D) shows the results for a human endometrial cancer cell line.

[Fig. 6]

Fig. 6 is a view showing a DR5-cB273 Fab complex structure.

[Fig. 7]

Fig. 7 is a view showing the interaction between DR5 and the H or L chain of cB273 Fab. A) is a view illustrating amino acid residues of the H chain of cB273 Fab which lie at a distance

of 4 Å or less from DR5 and *vice versa* as a stick model. Ile34, Glu36, Asp37, Gly38, Asp56, Leu57, Leu58, Phe59, Leu61, and Arg62 shown on the left side of the drawing are amino acid residues derived from DR5, and the respective amino acid residue numbers correspond to those in the amino acid sequence represented by SEQ ID NO: 23 in the Sequence Listing. Further, Phe33, Arg50, Asn52, Tyr54, Asn55, Phe59, Tyr101, Tyr102, Phe103, and Asp104 on the right side of the drawing are amino acid residues derived from the heavy chain of cB273, and the respective amino acid residue numbers are given by using a glutamic acid residue at position 20 of SEQ ID NO: 20 in the Sequence Listing as a starting point. B) is a view illustrating amino acid residues of the L chain of cB273 Fab which lie at a distance of 4 Å or less from DR5 and *vice versa* with some as a stick model and others as a ribbon model. Gly26, Glu36, Asp37, and Gly38 on the left side of the drawing are amino acid residues derived from DR5, and the respective amino acid residue numbers correspond to those in the amino acid sequence represented by SEQ ID NO: 23 in the Sequence Listing. Further, His31, Asn33, Val99, and Trp101 on the right side of the drawing are amino acid residues derived from the light chain of cB273, and the respective amino acid residue numbers are given by using an aspartic acid residue at position 21 of SEQ ID NO: 16 in the Sequence Listing as a starting point. The amino acid residues of DR5 which lie at a distance of 4 Å or less from

the Fab fragment of cB273 were a glycine residue at position 26, an isoleucine residue at position 34, a glutamic acid residue at position 36, an aspartic acid residue at position 37, a glycine residue at position 38, an aspartic acid residue at position 56, a leucine residue at position 57, a leucine residue at position 58, a phenylalanine residue at position 59, a leucine residue at position 61, and an arginine residue at position 62 of the amino acid sequence represented by SEQ ID NO: 23 in the Sequence Listing.

[Fig. 8-1]

Fig. 8-1 is a figure showing the binding activity of hB273 antibodies to human DR5 using Biacore, and shows measurement charts for the respective antibodies.

[Fig. 8-2]

Fig. 8-2 is a table showing the binding activity of hB273 antibodies to human DR5 using Biacore, and shows Kon, Koff, and KD values of the respective antibodies calculated using analysis software. Incidentally, the number given to each chart in Fig. 8-1 corresponds to the Entry No. of the table in Fig. 8-2.

[Fig. 9]

Fig. 9 is a figure showing the in vitro cytocidal activity of hB273 antibodies against Jurkat cells which are a human T lymphoma-derived cell line.

[Fig. 10-1]

Fig. 10-1 is a figure showing the binding activity of hB273 antibodies to human DR5 using Biacore, and shows measurement charts for the respective antibodies.

[Fig. 10-2]

Fig. 10-2 is a table showing the binding activity of hB273 antibodies to human DR5 using Biacore, and shows Kon, Koff, and KD values of the respective antibodies calculated using analysis software. Incidentally, the number given to each chart in Fig. 10-1 corresponds to the Entry No. in the table in Fig. 10-2.

[Fig. 11]

Fig. 11 is a figure showing the in vitro cytocidal activity of hB273 antibodies against Jurkat cells which are a human T lymphoma-derived cell line.

[Fig. 12-1]

Fig. 12-1 is a view the showing binding activity of CDR-modified hB273 antibodies to human DR5 using Biacore, and shows measurement charts for the respective antibodies.

[Fig. 12-2]

Fig. 12-2 is a table the showing binding activity of CDR-modified hB273 antibodies to human DR5 using Biacore, and shows Kon, Koff, and KD values of the respective antibodies calculated using analysis software. Incidentally, the number given to each chart in Fig. 12-1 corresponds to the Entry No. in the table in Fig. 12-2.

[Fig. 13-1]

Fig. 13-1 is a figure showing evaluation of thermal stability of CDR-modified hB273 antibodies using differential scanning calorimetry (DSC), and shows measurement charts for the respective antibodies.

[Fig. 13-2]

Fig. 13-2 is a figure showing evaluation of thermal stability of CDR-modified hB273 antibodies using differential scanning calorimetry (DSC), and shows measurement charts for the respective antibodies.

[Fig. 13-3]

Fig. 13-3 shows Tm values of the respective antibodies calculated from the charts shown in Figs. 13-1 and 13-2. Incidentally, the number given to each chart in Figs. 13-1 and 13-2 corresponds to the Entry No. in Fig. 13-3.

[Fig. 14]

Fig. 14 is a figure showing the in vitro cytocidal activities of CDR-modified hB273 antibodies against Jurkat cells which are a human T lymphoma-derived cell line.

[Fig. 15]

Fig. 15 is a view showing the caspase-3/7 activation effect and the in vitro cytocidal activity of a hB273_H2-1-NE/L1-NK antibody on human cancer cell lines. A) shows the results for a human colon cancer cell line HCT-15, and B) shows the results for a human glioblastoma cell line

U-87MG.

[Fig. 16]

Fig. 16 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human colon cancer cell line COLO 205.

[Fig. 17]

Fig. 17 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human pancreatic cancer cell line MIAPaCa-2.

[Fig. 18]

Fig. 18 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human glioblastoma cell line U-87MG.

[Fig. 19]

Fig. 19 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human lung cancer cell line NCI-H2122 (in combination with paclitaxel and carboplatin).

[Fig. 20]

Fig. 20 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human lung cancer cell line NCI-H460 (in combination with paclitaxel and carboplatin).

[Fig. 21]

Fig. 21 is a figure showing the in vivo antitumor activity

of a cB273 antibody in nude mice implanted with a human colon cancer cell line DLD-1 (in combination with CPT-11).

[Fig. 22]

Fig. 22 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human colon cancer cell line HCT-15 (in combination with CPT-11).

[Fig. 23]

Fig. 23 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human colon cancer cell line HCT-116 (in combination with CPT-11).

[Fig. 24]

Fig. 24 is a figure showing the in vivo antitumor activity of a cB273 antibody in nude mice implanted with a human melanoma cell line A375 (in combination with vinblastine).

[Fig. 25]

Fig. 25 is a figure showing a comparison of the in vivo antitumor activity in nude mice implanted with a human colon cancer cell line HCT-15 between a cB273 antibody and conatumumab.

[Fig. 26]

Fig. 26 is a figure showing a comparison of the in vivo antitumor activity in nude mice implanted with a human lung cancer cell line NCI-H1975 between a cB273 antibody and conatumumab.

[Fig. 27]

Fig. 27 is a figure showing the in vivo antitumor activity of a hB273_H2-1-NE/L1-NK antibody (denoted as “hB273” in the drawing) in nude mice implanted with a human colon cancer cell COLO 205.

[Fig. 28]

Fig. 28 is a figure showing the nucleotide sequence of a cDNA encoding a mouse antibody B273 heavy chain and the amino acid sequence of the mouse antibody B273 heavy chain.

[Fig. 29]

Fig. 29 is a figure showing the nucleotide sequence of a cDNA encoding a mouse antibody B273 light chain and the amino acid sequence of the mouse antibody B273 light chain.

[Fig. 30]

Fig. 30 is a figure showing a nucleotide sequence encoding a B273 chimera-type light chain and the amino acid sequence of the B273 chimera-type light chain.

[Fig. 31]

Fig. 31 is a figure showing a nucleotide sequence encoding a B273 chimera-type heavy chain and the amino acid sequence of the B273 chimera-type heavy chain.

[Fig. 32]

Fig. 32 is a figure showing a nucleotide sequence encoding a hB273_L1-type light chain and the amino acid sequence of the hB273_L1-type light chain.

[Fig. 33]

Fig. 33 is a figure showing a nucleotide sequence encoding a hB273_L2-type light chain and the amino acid sequence of the hB273_L2-type light chain.

[Fig. 34]

Fig. 34 is a figure showing a nucleotide sequence encoding a hB273_L3-type light chain and the amino acid sequence of the hB273_L3-type light chain.

[Fig. 35]

Fig. 35 is a figure showing a nucleotide sequence encoding a hB273_H1-type heavy chain and the amino acid sequence of the hB273_H1-type heavy chain.

[Fig. 36]

Fig. 36 is a figure showing a nucleotide sequence encoding a hB273_H2-type heavy chain and the amino acid sequence of the hB273_H2-type heavy chain.

[Fig. 37]

Fig. 37 is a figure showing a nucleotide sequence encoding a hB273_H3-type heavy chain and the amino acid sequence of the hB273_H3-type heavy chain.

[Fig. 38]

Fig. 38 is a figure showing a nucleotide sequence encoding a hB273_H1-1-type heavy chain and the amino acid sequence of the hB273_H1-1-type heavy chain.

[Fig. 39]

Fig. 39 is a figure showing a nucleotide sequence

encoding a hB273_H2-1-type heavy chain and the amino acid sequence of the hB273_H2-1-type heavy chain.

[Fig. 40]

Fig. 40 is a figure showing a nucleotide sequence encoding a hB273_H2-2-type heavy chain and the amino acid sequence of the hB273_H2-2-type heavy chain.

[Fig. 41]

Fig. 41 is a figure showing a nucleotide sequence encoding a hB273_H2-3-type heavy chain and the amino acid sequence of the hB273_H2-3-type heavy chain.

[Fig. 42]

Fig. 42 is a figure showing a nucleotide sequence encoding a hB273_H2-4-type heavy chain and the amino acid sequence of the hB273_H2-4-type heavy chain.

[Fig. 43]

Fig. 43 is a figure showing a nucleotide sequence encoding a hB273_H2-5-type heavy chain and the amino acid sequence of the hB273_H2-5-type heavy chain.

[Fig. 44]

Fig. 44 is a figure showing a nucleotide sequence encoding a hB273_L1-NE-type light chain and the amino acid sequence of the hB273_L1-NE-type light chain.

[Fig. 45]

Fig. 45 is a figure showing a nucleotide sequence encoding a hB273_L1-NF-type light chain and the amino acid

sequence of the hB273_L1-NF-type light chain.

[Fig. 46]

Fig. 46 is a figure showing a nucleotide sequence encoding a hB273_L1-NK-type light chain and the amino acid sequence of the hB273_L1-NK-type light chain.

[Fig. 47]

Fig. 47 is a figure showing a nucleotide sequence encoding a hB273_L1-NL-type light chain and the amino acid sequence of the hB273_L1-NL-type light chain.

[Fig. 48]

Fig. 48 is a figure showing a nucleotide sequence encoding a hB273_H2-1-NE-type heavy chain and the amino acid sequence of the hB273_H2-1-NE-type heavy chain.

[Fig. 49]

Fig. 49 is a figure showing the nucleotide sequence of a cDNA encoding a conatumumab light chain and the amino acid sequence of the conatumumab light chain.

[Fig. 50]

Fig. 50 is a figure showing the nucleotide sequence of a cDNA encoding a conatumumab heavy chain and the amino acid sequence of the conatumumab heavy chain.

[Fig. 51]

Fig. 51 is a figure showing the in vitro cytocidal activity of a hB273_H2-1-NE/hB273_L1-NK antibody against human cancer cell lines. A) shows the results for a human

stomach cancer cell line, B) shows the results for a human renal cancer cell line, C) shows the results for a human liver cancer cell line, and D) shows the results for a human fibrosarcoma cell line.

[Fig. 52]

Fig. 52 is a figure showing the in vivo antitumor activity of a hB273_H2-1-NE/hB273_L1-NK antibody (denoted as “hB273” in the drawing) in combination with 5-FU in nude mice implanted with a human colon cancer cell line HCT-15 and a comparison of the activity with conatumumab.

[Fig. 53]

Fig. 53 is a figure showing the in vivo antitumor activity of a hB273_H2-1-NE/hB273_L1-NK antibody (denoted as “hB273” in the drawing) in combination with paclitaxel in nude mice implanted with a human non-small cell lung cancer cell line NCI-H1975 and a comparison of the activity with conatumumab.

[Mode for Carrying Out the Invention]

The terms “cancer” and “tumor” as used herein are used with the same meaning.

The term “gene” as used herein includes not only DNA, but also mRNA thereof, cDNA and cRNA thereof.

The term “polynucleotide” as used herein is used with the same meaning as a “nucleic acid” and also includes DNA, RNA, probes, oligonucleotides, and primers.

The terms “polypeptide” and “protein” as used herein are

used without distinction.

The term “RNA fraction” as used herein refers to a fraction containing RNA.

The term “cell” as used herein also includes cells in an animal individual and cultured cells.

The term “malignant transformation of cells” as used herein refers to a state in which cells show abnormal proliferation, for example, cells lose their sensitivity to contact inhibition phenomenon, cells show anchorage-independent proliferation, and so on, and cells which show such abnormal proliferation are referred to as “cancer cells”.

The term “cell injury” as used herein refers to a state in which a pathological change is caused in cells in a form of some kind, and the cell injury is not limited to direct injury and includes all sorts of damage to the structure and function of cells such as DNA cleavage, base-dimer formation, chromosomal cleavage, damage to cell division machinery, and a decrease in various enzymatic activities.

The term “cytotoxic activity” as used herein refers to an activity of causing the above-described cell injury.

The term “death domain-containing receptor” (which includes Fas, TNFRI, DR3, DR4, DR5, and DR6, though is not limited thereto) as used herein refers to a receptor molecule having an apoptotic signal transduction region called a “death

domain" showing homology with a Drosophila suicide gene, reaper, in an intracellular domain.

The term "functional fragment of an antibody" as used herein refers to a partial fragment of an antibody having an antigen binding activity and includes Fab, $F(ab')_2$, scFv, and the like. The term also includes Fab' which is a monovalent fragment in a variable region of an antibody obtained by treating $F(ab')_2$ under reducing conditions. However, the term is not limited to these molecules as long as the fragment has a binding affinity for an antigen. Further, these functional fragments include not only a fragment obtained by treating a full-length molecule of an antibody protein with an appropriate enzyme, but also a protein produced in an appropriate host cell using a genetically modified antibody gene.

The term "Fab'" as used herein refers to a monovalent fragment in a variable region of an antibody obtained by treating $F(ab')_2$ under reducing conditions as described above. However, Fab' produced using a genetically modified antibody gene is also included within the Fab' of the invention.

The term "single-chain variable fragment antibody" as used herein is used with the same meaning as a single-chain Fv (scFv).

The term "epitope" as used herein refers to a partial peptide or a partial tertiary structure of an antigen to which a specific antibody binds. The epitope which is a partial

peptide of an antigen can be determined by methods well known to those skilled in the art such as an immunoassay, and for example, the following method can be employed. First, various partial structures of an antigen are produced. In the production of the partial structures, a known oligopeptide synthesis technique can be used. For example, a series of polypeptides having appropriately reduced lengths obtained by sequentially shortening the antigen from the C terminus or N terminus are produced using a genetic recombination technique known to those skilled in the art. Thereafter, the reactivity of an antibody against these polypeptides is examined and a recognition site is roughly determined. Then, peptides having shorter lengths are synthesized and the reactivity with these peptides is examined, whereby the epitope can be determined. Further, the epitope which is a partial tertiary structure of an antigen binding to a specific antibody can be determined by specifying the amino acid residues of the antigen which lie adjacent to the antibody by X-ray structural analysis.

The term “antibodies which bind to the same epitope” as used herein refers to different antibodies which bind to a common epitope. If a second antibody binds to a partial peptide or a partial tertiary structure to which a first antibody binds, it can be determined that the first antibody and the second antibody bind to the same epitope. Further, by confirming that the second antibody competes with the first antibody for the

binding to an antigen (that is, the second antibody inhibits the binding between the first antibody and the antigen), it can be determined that the first antibody and the second antibody bind to the same epitope even if the specific epitope sequence or structure has not been determined. Further, when the first antibody and the second antibody bind to the same epitope and also the first antibody has a special effect such as an apoptosis-inducing activity, it can be expected that the second antibody also has the same activity.

The term "CDR" as used herein refers to a complementarity determining region (CDR), and it is known that each heavy and light chain of an antibody molecule has three complementarity determining regions (CDRs). The CDR is also called the hypervariable domain, and is present in a variable region of each heavy and light chain of an antibody. It is a site which has unusually high variability in its primary structure, and there are three separate CDRs in the primary structure of each heavy and light polypeptide chain. In this specification, as for the CDRs of an antibody, the CDRs of the heavy chain are represented by CDRH1, CDRH2, and CDRH3 from the amino-terminal side of the amino acid sequence of the heavy chain, and the CDRs of the light chain are represented by CDRL1, CDRL2, and CDRL3 from the amino-terminal side of the amino acid sequence of the light chain. These sites are proximate to one another in the tertiary structure and determine the specificity for

an antigen to which the antibody binds.

The term “secondary antibody” as used herein refers to an antibody which binds specifically to an antibody molecule, thereby crosslinking the antibody molecules.

The phrase “hybridization is performed under stringent conditions” as used herein refers to a process in which hybridization is performed under conditions under which identification can be achieved by performing hybridization at 68°C in a commercially available hybridization solution ExpressHyb Hybridization Solution (manufactured by Clontech, Inc.) or by performing hybridization at 68°C in the presence of 0.7 to 1.0 M NaCl using a filter having DNA immobilized thereon, followed by performing washing at 68°C using 0.1 to 2 x SSC solution (1 x SSC solution is composed of 150 mM NaCl and 15 mM sodium citrate) or under conditions equivalent thereto.

The term “several amino acids” in the description of “an amino acid sequence including a substitution, deletion, or addition of one to several amino acids” as used herein refers to an arbitrary number of amino acid residues selected from 2 to 10. More specifically, when 10 or fewer amino acids, 5 to 6 or fewer amino acids, or 2 to 3 or fewer amino acids are substituted, deleted or added, the description of “an amino acid sequence including a substitution, deletion, or addition of several amino acids” is used.

The description of, for example, “a heavy chain variable region having an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 34” as used herein is used with the same meaning as the description of “a heavy chain variable region sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 34”. Further, the description of, for example, “a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 34” is used with the same meaning as the description of “a heavy chain sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 34”.

1. Regarding apoptosis-related gene

An antibody according to the invention is required to bind to a specific antigen and exhibit a cytotoxic activity via the antigen. Further, it is necessary to select the antigen specifically present in tumor cells to prevent normal cells from being killed. One example of such an antigen group can include tumor necrosis factor (hereinafter, referred to as “TNF” in the specification) related apoptosis-inducing ligand (hereinafter, referred to as “TRAIL” in the specification) receptor groups. TRAIL is a member of the TNF family of proteins and includes Fas ligands and TNF-α (Wiley SR, et al., Immunity 1995 Dec; 3 (6): 673-82). These proteins are strong apoptosis-inducing factors.

Receptors for these TNF family proteins are

characterized by cysteine-rich repeat sequences in the extracellular domain. Among these, Fas which is a receptor for Fas ligands, and TNF receptor I (hereinafter, referred to as “TNFRI” in the specification) which is a receptor for TNFα have, in an intracellular domain, a region essential for apoptotic signal transduction called the “death domain” which is a region showing homology with the Drosophila suicide gene, reaper, (Golstein, P., et al., (1995) Cell. 81, 185-186; and White, K, et al., (1994) Science 264, 677-683), and are collectively called death domain-containing receptors.

Five receptors for TRAIL have been identified, and among them, two receptors (DR4 (TRAIL-R1) and DR5 (TRAIL-R2)) are capable of transducing an apoptotic signal, and the other three receptors (DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG)) do not transduce an apoptotic signal. Similarly to Fas and TNFRI, both DR4 and DR5 include a death domain in an intracellular segment and transduce an apoptotic signal via a pathway containing a Fas-associated death domain protein (hereinafter referred to as “FADD” in the specification) and caspase 8 (Chaudhary PM, et al., Immunity 1997 Dec; 7 (6): 813-20; and Schneider P, et al. Immunity 1997 Dec; 7 (6): 821-30). For the Fas, TNFRI, DR4, or DR5 described above, it is known that an antibody which binds to any of these molecules and functions as an agonist exhibits an apoptosis-inducing activity against cells bearing the

molecule on the cell surface (Journal of Cellular Physiology, 209: 1221-1028 (2006); Leukemia, Apl; 21 (4): 805-812 (2007); Blood, 99: 1666-1675 (2002); and Cellular Immunology, Jan; 153 (1): 184-193 (1994)). The pharmacological effect of the above-described agonistic antibody is enhanced by crosslinking with a secondary antibody or an effector cell (Journal of Immunology, 149: 3166-3173 (1992); and European Journal of Immunology, Oct; 23 (10): 2676-2681 (1993)).

The nucleotide sequence of a human DR5 (death receptor 5) gene and the amino acid sequence thereof have been registered as GI:22547118 (Accession No: NM_147187) in GenBank. Incidentally, a nucleotide sequence encoding a protein, which has an amino acid sequence including a substitution, deletion, or addition of one to several amino acids in the amino acid sequence of DR5 and also has a biological activity equivalent to that of DR5 is also included within the meaning of the term “nucleotide sequence of the DR5 gene”. Further, a protein which has an amino acid sequence including a substitution, deletion, or addition of one to several amino acids in the amino acid sequence of DR5 and also has a biological activity equivalent to that of DR5 is also included within the meaning of the term “DR5”.

2. Production of anti-DR5 antibody

The antibody against DR5 of the invention can be obtained

by immunizing an animal with DR5 or an arbitrary polypeptide selected from the amino acid sequence of DR5 and collecting and purifying the antibody produced in vivo according to a common procedure. The biological species of the DR5 to be used as an antigen is not limited to human, and an animal can be immunized with DR5 derived from an animal other than humans such as a mouse or a rat. In this case, by examining the cross-reactivity between an antibody which binds to the obtained heterologous DR5 and human DR5, an antibody applicable to a human disease can be selected.

Further, a monoclonal antibody can be obtained by fusing antibody-producing cells which produce an antibody against DR5 with myeloma cells to establish a hybridoma according to a known method (for example, Kohler and Milstein, Nature, (1975) 256, pp. 495-497, Kennet, R. ed., Monoclonal Antibodies, pp. 365-367, Plenum Press, N.Y. (1980)).

Incidentally, DR5 to be used as an antigen can be obtained by genetic engineering to cause a host cell to express a DR5 gene.

Specifically, a vector capable of expressing a DR5 gene is produced, and the resulting vector is transfected into a host cell to express the gene, and then the expressed DR5 is purified. Hereinafter, a method of obtaining an antibody against DR5 will be specifically described.

(1) Preparation of antigen

Examples of the antigen to be used for producing the anti-DR5 antibody include DR5, a polypeptide comprising a partial amino acid sequence containing at least 6 consecutive amino acids of DR5, and a derivative obtained by adding a given amino acid sequence or carrier thereto.

DR5 can be purified directly from human tumor tissues or tumor cells and used. Further, DR5 can be obtained by synthesizing it in vitro or by causing a host cell to produce it by genetic engineering.

With respect to genetic engineering, specifically, DR5 cDNA is integrated into a vector capable of expressing DR5 cDNA and DR5 is synthesized in a solution containing an enzyme, a substrate, and an energy substance required for transcription and translation, or another prokaryotic or eucaryotic host cell is transformed to express DR5, whereby the antigen can be obtained.

Further, the antigen can also be obtained as a secretory protein by expressing a fusion protein obtained by joining the extracellular domain of DR5, which is a membrane protein, to the constant region of an antibody in an appropriate host-vector system.

DR5 cDNA can be obtained by, for example, the so-called PCR method in which a polymerase chain reaction (hereinafter referred to as “PCR”) is performed using a cDNA library containing DR5 cDNA as a template and primers which

specifically amplify DR5 cDNA (see Saiki, R. K., et al., Science, (1988) 239, pp. 487-489).

As for the system for in vitro synthesis of the polypeptide, the Rapid Translation System (RTS) manufactured by Roche Diagnostics, Inc. can be exemplified, but it is not limited thereto.

Examples of the prokaryotic host cell include Escherichia coli and Bacillus subtilis. In order to transform the host cell with a target gene, the host cell is transformed using a plasmid vector containing a replicon, i.e., a replication origin derived from a species compatible with the host, and a regulatory sequence. Further, the vector preferably has a sequence capable of imposing phenotypic selectivity on the transformed cell.

Examples of the eucaryotic host cell include vertebrate cells, insect cells, and yeast cells. As the vertebrate cells, for example, dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61); and the like are often used, however, they are not limited thereto.

The thus obtained transformant can be cultured according to a common procedure, and by the culturing of the transformant,

a target polypeptide is produced intracellularly or extracellularly.

A suitable medium to be used for the culturing can be selected from various commonly used culture media depending on the employed host cell. If Escherichia coli is employed, for example, LB medium supplemented with an antibiotic such as ampicillin or IPMG, as needed, can be used.

A recombinant protein produced intracellularly or extracellularly by the transformant through such culturing can be separated and purified by any of various known separation methods utilizing a physical or chemical property of the protein.

Specific examples of the methods include treatment with a common protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, and affinity chromatography, dialysis, and a combination thereof.

Further, by attaching a tag of six histidine residues to a recombinant protein to be expressed, the protein can be efficiently purified with a nickel affinity column. Alternatively, by attaching an IgG Fc region to a recombinant protein to be expressed, the protein can be efficiently purified with a protein A column.

By combining the above-described methods, a large amount

of a target polypeptide can be easily produced in high yield and high purity.

(2) Production of anti-DR5 monoclonal antibody

Examples of the antibody which specifically binds to DR5 include a monoclonal antibody which specifically binds to DR5, and a method of obtaining the antibody is as described below.

The production of a monoclonal antibody generally requires the following operational steps of:

(a) purifying a biopolymer to be used as an antigen;

(b) preparing antibody-producing cells by immunizing an animal by injection of the antigen, collecting the blood, assaying its antibody titer to determine when the spleen is to be excised;

(c) preparing myeloma cells (hereinafter referred to as "myeloma");

(d) fusing the antibody-producing cells with the myeloma;

(e) screening a group of hybridomas producing a target antibody;

(f) dividing the hybridomas into single cell clones (cloning);

(g) optionally, culturing the hybridoma or rearing an animal implanted with the hybridoma for producing a large amount of a monoclonal antibody;

(h) examining the thus produced monoclonal antibody for

biological activity and binding specificity, or assaying the same for properties as a labeled reagent; and the like.

Hereinafter, the method of producing a monoclonal antibody will be described in detail following the above steps, however, the method is not limited thereto, and, for example, antibody-producing cells other than spleen cells and myeloma can be used.

(a) Purification of antigen

As the antigen, DR5 prepared by the method as described above or a partial peptide thereof can be used.

Further, a membrane fraction prepared from recombinant cells expressing DR5 or the recombinant cells expressing DR5 themselves, and also a partial peptide of the protein of the invention chemically synthesized by a method known to those skilled in the art can also be used as the antigen.

(b) Preparation of antibody-producing cells

The antigen obtained in step (a) is mixed with an adjuvant such as Freund's complete or incomplete adjuvant, or aluminum potassium sulfate and the resulting mixture is used as an immunogen to immunize an experimental animal. As the experimental animal, any animal used in a known hybridoma production method can be used without any trouble. Specifically, for example, a mouse, a rat, a goat, sheep, cattle, a horse, or the like can be used. However, from the viewpoint of ease of availability of myeloma cells to be fused with the

extracted antibody-producing cells, a mouse or a rat is preferably used as the animal to be immunized.

Further, the strain of mouse or rat to be used is not particularly limited, and in the case of a mouse, for example, various strains such as A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BL, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, R III, SJL, SWR, WB, and 129 can be used, and in the case of a rat, for example, Wistar, Low, Lewis, Sprague, Dawley, ACI, BN, Fischer, and the like can be used.

Among these, in consideration of compatibility of fusing with myeloma cells as described below, in the case of a mouse, BALB/c strain, and in the case of a rat, Wistar and Low strains are particularly preferred as the animal to be immunized.

Further, in consideration of antigenic homology between humans and mice, it is also preferred to use a mouse having decreased biological function to remove autoantibodies, that is, a mouse with an autoimmune disease.

The age of the mouse or rat at the time of immunization is preferably 5 to 12 weeks of age, more preferably 6 to 8 weeks of age.

In order to immunize an animal with DR5 or a recombinant protein thereof, for example, a known method described in detail in, for example, Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental

Immunochemistry, Charles C Thomas Publisher Springfield, Illinois (1964), or the like can be used.

Spleen cells or lymphocytes including antibody-producing cells are aseptically removed from the immunized animal. At this time, the antibody titer is measured, and if an animal having a sufficiently increased antibody titer is used as a supply source of the antibody-producing cells, the subsequent procedure can be carried out more efficiently.

Examples of the method of measuring the antibody titer to be used here include an RIA method and an ELISA method, but the method is not limited thereto.

The separation of the antibody-producing cells from the spleen cells or lymphocytes of the immunized animal can be carried out according to a known method (for example, Kohler et al., Nature (1975), 256, p. 495; Kohler et al., Eur. J. Immunol. (1977), 6, p. 511; Milstein et al., Nature (1977), 266, p. 550; Walsh, Nature (1977), 266, p. 495).

(c) Myeloma cells (hereinafter referred to as "myeloma")

The myeloma cells to be used for cell fusion are not particularly limited and suitable cells can be selected from known cell lines. However, in consideration of convenience when a hybridoma is selected from fused cells, it is preferred to use an HGPRT (hypoxanthine-guanine phosphoribosyl transferase) deficient strain whose selection procedure has been established.

More specifically, examples of the HGPRT-deficient strain include X63-Ag8 (X63), NS1-ANS/1 (NS1), P3X63-Ag8.U1 (P3U1), X63-Ag8.653 (X63.653), SP2/0-Ag14 (SP2/0), MPC11-45.6TG1.7 (45.6TG), F0, S149/5XXO, and BU.1 derived from mice; 210.RSY3.Ag.1.2.3 (Y3) derived from rats; and U266AR (SKO-007), GM1500·GTG-A12 (GM1500), UC729-6, LICR-LOW-HMy2 (HMy2) and 8226AR/NIP4-1 (NP41) derived from humans.

(d) Cell fusion

Fusion between the antibody-producing cells and the myeloma cells can be appropriately performed according to a known method (Weir, D. M. Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher, Spigfield, Illinois (1964), etc.), under conditions such that the survival rate of cells is not excessively reduced.

As such a method, for example, a chemical method in which the antibody-producing cells and the myeloma cells are mixed in a solution containing a polymer such as polyethylene glycol at a high concentration, a physical method using electric stimulation, or the like can be used.

(e) Selection of a group of hybridomas

The method of selecting hybridomas obtained by the above-described cell fusion is not particularly limited. Usually, an HAT (hypoxanthine, aminopterin, thymidine)

selection method (Kohler et al., Nature (1975), 256, p. 495; Milstein et al., Nature (1977), 266, p. 550) is used.

This method is effective when hybridomas are obtained using the myeloma cells of an HGPRT-deficient strain which cannot survive in the presence of aminopterin.

That is, by culturing unfused cells and hybridomas in HAT medium, only hybridomas resistant to aminopterin are selectively allowed to survive and proliferate.

(f) Division into single cell clone (cloning)

As a cloning method for hybridomas, a known method such as a methylcellulose method, a soft agarose method, or a limiting dilution method can be used (see, for example, Barbara, B. M. and Stanley, M. S.: Selected Methods in Cellular Immunology, W. H. Freeman and Company, San Francisco (1980)). Among these methods, particularly, a three-dimensional culture method such as a methylcellulose method is preferred. For example, the group of hybridomas produced by cell fusion are suspended in a methylcellulose medium such as ClonaCell-HY Selection Medium D (manufactured by StemCell Technologies, inc., #03804) and cultured. Then, the formed hybridoma colonies are collected, whereby monoclonal hybridomas can be obtained. The collected respective hybridoma colonies are cultured, and a hybridoma which has been confirmed to have a stable antibody titer in an obtained hybridoma culture supernatant is selected as a DR5 monoclonal antibody-producing

hybridoma strain.

Examples of the thus established hybridoma strain include DR5 hybridoma B273. Incidentally, in this specification, an antibody produced by the hybridoma B273 is referred to as “B273 antibody” or simply “B273”. The heavy chain of the B273 antibody has an amino acid sequence represented by SEQ ID NO: 8 in the Sequence Listing. Further, the light chain of the B273 antibody has an amino acid sequence represented by SEQ ID NO: 10 in the Sequence Listing. Incidentally, in the heavy chain amino acid sequence represented by SEQ ID NO: 8 in the Sequence Listing, an amino acid sequence comprising amino acid residues 1 to 19 is a signal sequence, an amino acid sequence comprising amino acid residues 20 to 141 is a variable region, and an amino acid sequence comprising amino acid residues 142 to 465 is a constant region. Further, in the light chain amino acid sequence represented by SEQ ID NO: 10 in the Sequence Listing, an amino acid sequence comprising amino acid residues 1 to 19 is a signal sequence, an amino acid sequence comprising amino acid residues 20 to 133 is a variable region, and an amino acid sequence comprising amino acid residues 134 to 238 is a constant region.

The heavy chain amino acid sequence represented by SEQ ID NO: 8 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 7 in the Sequence Listing. In the nucleotide sequence represented by SEQ ID NO: 7 in the

Sequence Listing, a nucleotide sequence comprising nucleotides 1 to 57 encodes the heavy chain signal sequence of the antibody, a nucleotide sequence comprising nucleotides 58 to 423 encodes the heavy chain variable region of the antibody, and a nucleotide sequence comprising nucleotides 424 to 1395 encodes the heavy chain constant region of the antibody.

The light chain amino acid sequence represented by SEQ ID NO: 10 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 9 in the Sequence Listing. In the nucleotide sequence represented by SEQ ID NO: 9 in the Sequence Listing, a nucleotide sequence comprising nucleotides 1 to 57 encodes the light chain signal sequence of the antibody, a nucleotide sequence comprising nucleotides 58 to 399 encodes the light chain variable region of the antibody, and a nucleotide sequence comprising nucleotides 400 to 714 encodes the light chain constant region of the antibody.

(g) Preparation of monoclonal antibody by culturing hybridoma

By culturing the thus selected hybridoma, a monoclonal antibody can be efficiently obtained. However, prior to culturing, it is preferred to perform screening of a hybridoma which produces a target monoclonal antibody.

In such screening, a known method can be employed.

The measurement of the antibody titer in the invention can be carried out by, for example, an ELISA method explained

in item (b) described above.

The hybridoma obtained by the method described above can be stored in a frozen state in liquid nitrogen or in a freezer at -80°C or below.

After completion of cloning, the medium is changed from HT medium to a normal medium, and the hybridoma is cultured.

Large-scale culture is performed by rotation culture using a large culture bottle or by spinner culture. From the supernatant obtained by the large-scale culture, a monoclonal antibody which specifically binds to the protein of the invention can be obtained by purification using a method known to those skilled in the art such as gel filtration.

Further, the hybridoma is injected into the abdominal cavity of a mouse of the same strain as the hybridoma (for example, the above-described BALB/c) or a Nu/Nu mouse to proliferate the hybridoma, whereby the ascites containing a large amount of the monoclonal antibody of the invention can be obtained.

In the case where the hybridoma is administered in the abdominal cavity, if a mineral oil such as 2,6,10,14-tetramethyl pentadecane (pristane) is administered prior to (3 to 7 days before) administration of the hybridoma, a larger amount of the ascites can be obtained.

For example, an immunosuppressant is previously injected into the abdominal cavity of a mouse of the same strain as the

hybridoma to inactivate T cells. 20 days thereafter, 10^6 to 10^7 hybridoma clone cells are suspended in a serum-free medium (0.5 ml), and the suspension is injected into the abdominal cavity of the mouse. In general, when the abdomen is expanded and filled with the ascites, the ascites is collected from the mouse. By this method, the monoclonal antibody can be obtained at a concentration which is about 100 times or more higher than that in the culture solution.

The monoclonal antibody obtained by the above-described method can be purified by a method described in, for example, Weir, D. M.: Handbook of Experimental Immunology Vol. I, II, III, Blackwell Scientific Publications, Oxford (1978).

The thus obtained monoclonal antibody has high antigen specificity for DR5.

(h) Assay of monoclonal antibody

The isotype and subclass of the thus obtained monoclonal antibody can be determined as follows.

First, examples of the identification method include an Ouchterlony method, an ELISA method and an RIA method.

An Ouchterlony method is simple, but when the concentration of the monoclonal antibody is low, a condensation operation is required.

On the other hand, when an ELISA method or an RIA method is used, by directly reacting the culture supernatant with an antigen-adsorbed solid phase and using antibodies

corresponding to various types of immunoglobulin isotypes and subclasses as secondary antibodies, the isotype and subclass of the monoclonal antibody can be identified.

In addition, as a simpler method, a commercially available identification kit (for example, Mouse Typer Kit manufactured by Bio-Rad Laboratories, Inc.) or the like can also be used.

Further, the quantitative determination of a protein can be performed by the Folin Lowry method and a method of calculation based on the absorbance at 280 nm [1.4 (OD 280) = Immunoglobulin 1 mg/ml].

Further, even when the monoclonal antibody is separately and independently obtained by performing again the steps of (a) to (h) in the above (2), it is possible to obtain an antibody having a cytotoxic activity equivalent to that of B273. As one example of such an antibody, an antibody which binds to the same epitope as the B273 antibody can be exemplified. If a newly produced monoclonal antibody binds to a partial peptide or a partial tertiary structure to which the B273 antibody binds, it can be determined that the monoclonal antibody binds to the same epitope as the B273 antibody. Further, by confirming that the monoclonal antibody competes with the B273 antibody for binding to DR5 (that is, the monoclonal antibody inhibits the binding between the B273 antibody and DR5), it can be determined that the monoclonal antibody binds to the same epitope as the

B273 antibody even if the specific epitope sequence or structure has not been determined. In the case where the monoclonal antibody binds to the same epitope as the B273 antibody, the monoclonal antibody is strongly expected to have a cytotoxic activity equivalent to that of B273.

Further, as shown in Example 4, it is possible to specify an amino acid residue on the side of DR5 which lies adjacent to a Fab fragment of an antibody from the data of X-ray diffraction of a complex between the Fab fragment and DR5. Specifically, in the case where a Fab fragment derived from an arbitrary antibody lies adjacent to a glycine residue at position 26, an isoleucine residue at position 34, a glutamic acid residue at position 36, an aspartic acid residue at position 37, a glycine residue at position 38, an aspartic acid residue at position 56, a leucine residue at position 57, a leucine residue at position 58, a phenylalanine residue at position 59, a leucine residue at position 61, and an arginine residue at position 62 of an amino acid sequence represented by SEQ ID NO: 23 in the Sequence Listing, at a distance of 4 Å or less, it can be determined that the antibody has specificity for the same epitope as B273.

(3) Other antibodies

The antibody of the invention includes not only the above-described monoclonal antibody against DR5 but also a recombinant antibody obtained by artificial modification for

the purpose of decreasing heterologous antigenicity in humans such as a chimeric antibody, a humanized antibody and a human antibody. These antibodies can be produced using a known method.

As the chimeric antibody, an antibody in which antibody variable and constant regions are derived from different species, for example, a chimeric antibody in which a mouse- or rat-derived antibody variable region is joined to a human-derived constant region can be exemplified (see Proc. Natl. Acad. Sci. USA, 81, 6851-6855, (1984)). A chimeric antibody derived from a mouse anti-human DR5 antibody B273 is an antibody comprising a heavy chain containing a heavy chain variable region having an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 20 and a light chain containing a light chain variable region having an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 16, and may have an arbitrary constant region. As one example of such a chimeric antibody, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 20 in the Sequence Listing and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 16 can be exemplified. Incidentally, in the heavy chain sequence represented by SEQ ID NO: 20 in the Sequence Listing, an amino acid sequence comprising amino acid residues 1 to 19 is a signal sequence,

an amino acid sequence comprising amino acid residues 20 to 141 is a variable region, and an amino acid sequence comprising amino acid residues 142 to 471 is a constant region. Further, in the light chain amino acid sequence represented by SEQ ID NO: 16 in the Sequence Listing, an amino acid sequence comprising amino acid residues 1 to 20 is a signal sequence, an amino acid sequence comprising amino acid residues 21 to 134 is a variable region, and an amino acid sequence comprising amino acid residues 135 to 239 is a constant region.

The heavy chain amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 19 in the Sequence Listing. In the nucleotide sequence represented by SEQ ID NO: 19 in the Sequence Listing, a nucleotide sequence comprising nucleotides 1 to 57 encodes the heavy chain signal sequence of the antibody, a nucleotide sequence comprising nucleotides 58 to 423 encodes the heavy chain variable region of the antibody, and a nucleotide sequence comprising nucleotides 424 to 1413 encodes the heavy chain constant region of the antibody.

The light chain amino acid sequence represented by SEQ ID NO: 16 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 15 in the Sequence Listing. In the nucleotide sequence represented by SEQ ID NO: 15 in the Sequence Listing, a nucleotide sequence comprising nucleotides 1 to 60 encodes the light chain signal sequence

of the antibody, a nucleotide sequence comprising nucleotides 61 to 402 encodes the light chain variable region of the antibody, and a nucleotide sequence comprising nucleotides 403 to 717 encodes the light chain constant region of the antibody.

As the humanized antibody, an antibody obtained by integrating only the complementarity determining regions (CDRs) into a human-derived antibody (see Nature (1986) 321, pp. 522-525), and an antibody obtained by grafting a part of the amino acid residues of the framework as well as the CDR sequences to a human antibody by a CDR-grafting method (WO 90/07861) can be exemplified.

However, the humanized antibody derived from the B273 antibody is not limited to a specific humanized antibody as long as the humanized antibody has all 6 types of CDR sequences of B273 and has the activity of inducing apoptosis in cells. Incidentally, the heavy chain variable region of the B273 antibody has CDRH1 (GYFMN) consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 (RFNPYNGDTFYNQKFKG) consisting of an amino acid sequence represented by SEQ ID NO: 83, and CDRH3 (SAYYFDSGGYFDY) consisting of an amino acid sequence represented by SEQ ID NO: 84. Further, the light chain variable region of the B273 antibody has CDRL1 (RSSQSLVHSNGNTYLH) consisting of an amino acid sequence represented by SEQ ID NO: 79 in the Sequence Listing, CDRL2 (KVSNRFS) consisting of an amino acid sequence

represented by SEQ ID NO: 80, and CDRL3 (SQSTHVPWT) consisting of an amino acid sequence represented by SEQ ID NO: 81.

Further, a sequence including the substitution, deletion, or addition of one to several amino acid residues in one of the above-described CDRs can be used as a CDR sequence which a CDR-modified antibody derived from the B273 antibody has. Examples of the sequence including a substitution of one amino acid residue in CDRL1 include a sequence (RSSQSLVHSNENTYLH) consisting of an amino acid sequence represented by SEQ ID NO: 85 in the Sequence Listing, a sequence (RSSQSLVHSNFNTYLH) consisting of an amino acid sequence represented by SEQ ID NO: 86, a sequence (RSSQSLVHSNKNTYLH) consisting of an amino acid sequence represented by SEQ ID NO: 87, and a sequence (RSSQSLVHSNLNTYLH) consisting of an amino acid sequence represented by SEQ ID NO: 88. Further, examples of the sequence including a substitution of one amino acid residue in CDRH2 include a sequence (RFNPYNEDTFYNQKFKG) consisting of an amino acid sequence represented by SEQ ID NO: 89.

In general, the deamidation of asparagine in a protein proceeds through the formation of a transition state of cyclic succinimide between the asparagine and an adjacent amino acid on the C-terminal side (Geiger, T. and Clarke, S. (1987) Deamidation, Isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem.

262, 785-794). A rate-limiting factor for the formation of a transition state of cyclic succinimide is the size of a side chain of the adjacent amino acid, and therefore, glycine which has the smallest side chain can achieve the fastest deamidation rate. On the other hand, by substituting the adjacent group on the C-terminal side with an amino acid having a large side chain, the deamidation rate can be suppressed. The B273 antibody has a -N-G- (asparagine-glycine) sequence which is susceptible to deamidation in CDRL1 and CDRH2. Therefore, the present inventors produced point mutants in which the adjacent group was changed from glycine to lysine, phenylalanine, leucine, or glutamic acid, each of which has a larger side chain than glycine. That is, in CDRH2, the -N-G- (asparagine-glycine) sequence is mutated to a -N-E- (asparagine-glutamic acid) sequence, and in CDRL1, the -N-G- (asparagine-glycine) sequence is mutated to a -N-L- (asparagine-leucine) sequence, a -N-F- (asparagine-phenylalanine) sequence, a -N-K- (asparagine-lysine) sequence, or a -N-E- (asparagine-glutamic acid) sequence, whereby the deamidation of the antibody is suppressed.

As an example of an antibody having the above-described CDRs, an antibody containing a heavy chain variable region having CDRH1 consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 consisting of

an amino acid sequence represented by SEQ ID NO: 83, and CDRH3 consisting of an amino acid sequence represented by SEQ ID NO: 84, and a light chain variable region having CDRL1 consisting of an amino acid sequence represented by SEQ ID NO: 79 in the Sequence Listing, CDRL2 consisting of an amino acid sequence represented by SEQ ID NO: 80, and CDRL3 consisting of an amino acid sequence represented by SEQ ID NO: 81; an antibody containing a heavy chain variable region having CDRH1 consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 consisting of an amino acid sequence represented by SEQ ID NO: 89, and CDRH3 consisting of an amino acid sequence represented by SEQ ID NO: 84, and a light chain variable region having CDRL1 consisting of an amino acid sequence represented by SEQ ID NO: 85 in the Sequence Listing, CDRL2 consisting of an amino acid sequence represented by SEQ ID NO: 80, and CDRL3 consisting of an amino acid sequence represented by SEQ ID NO: 81; an antibody containing a heavy chain variable region having CDRH1 consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 consisting of an amino acid sequence represented by SEQ ID NO: 89, and CDRH3 consisting of an amino acid sequence represented by SEQ ID NO: 84, and a light chain variable region having CDRL1 consisting of an amino acid sequence represented by SEQ ID NO: 86 in the Sequence Listing, CDRL2 consisting of an amino acid sequence represented by SEQ ID NO: 80, and CDRL3

consisting of an amino acid sequence represented by SEQ ID NO: 81; an antibody containing a heavy chain variable region having CDRH1 consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 consisting of an amino acid sequence represented by SEQ ID NO: 89, and CDRH3 consisting of an amino acid sequence represented by SEQ ID NO: 84, and a light chain variable region having CDRL1 consisting of an amino acid sequence represented by SEQ ID NO: 87 in the Sequence Listing, CDRL2 consisting of an amino acid sequence represented by SEQ ID NO: 80, and CDRL3 consisting of an amino acid sequence represented by SEQ ID NO: 81; and an antibody containing a heavy chain variable region having CDRH1 consisting of an amino acid sequence represented by SEQ ID NO: 82 in the Sequence Listing, CDRH2 consisting of an amino acid sequence represented by SEQ ID NO: 89, and CDRH3 consisting of an amino acid sequence represented by SEQ ID NO: 84, and a light chain variable region having CDRL1 consisting of an amino acid sequence represented by SEQ ID NO: 88 in the Sequence Listing, CDRL2 consisting of an amino acid sequence represented by SEQ ID NO: 80, and CDRL3 consisting of an amino acid sequence represented by SEQ ID NO: 81 can be exemplified.

As an example of the humanized antibody of the mouse antibody B273 (including a CDR-modified antibody), an arbitrary combination of a heavy chain containing a heavy chain variable region comprising an amino acid sequence comprising

amino acid residues 20 to 141 of any one of SEQ ID NOS: 34, 36, 38, 40, 42, 44, 46, 48, 50, and 70 in the Sequence Listing with a light chain containing a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of any one of SEQ ID NOS: 28, 30, 32, 52, 58, 62, and 66 can be exemplified.

As a preferred combination, an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 34 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 34 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 30; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 34 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 32; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 36 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ

ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 36 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 30; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 36 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 32; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 38 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 38 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 30; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 38 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 32; an antibody characterized by containing a heavy chain variable region comprising an amino

acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 40 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 42 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 44 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 46 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 48 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 50 and a light chain variable

region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 52; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 58; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 62; and an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 66 can be exemplified.

As a more preferred combination, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 34 and a light chain having an amino acid sequence comprising amino acid residues 21 to

239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 34 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 30; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 34 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 32; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 36 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 36 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 30; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 36 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 32; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 38 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 38 and

a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 30; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 38 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 32; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 40 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 42 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 44 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 46 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 48 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino

acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 50 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 52; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 58; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 62; and an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 66 can be exemplified.

As a further more preferred combination, an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 42 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 28; an antibody characterized

by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 52; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 58; an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 62; and an antibody characterized by containing a heavy chain variable region comprising an amino acid sequence comprising amino acid residues 20 to 141 of SEQ ID NO: 70 and a light chain variable region comprising an amino acid sequence comprising amino acid residues 21 to 134 of SEQ ID NO: 66 can be exemplified.

As a most preferred combination, an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 42 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 28; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to

471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 52; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 58; an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 62; and an antibody comprising a heavy chain having an amino acid sequence comprising amino acid residues 20 to 471 of SEQ ID NO: 70 and a light chain having an amino acid sequence comprising amino acid residues 21 to 239 of SEQ ID NO: 66 can be exemplified.

By combining a sequence having a high homology with the above-described heavy chain amino acid sequence with a sequence having a high homology with the above-described light chain amino acid sequence, it is possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies. The homology is generally a homology of 80% or more, preferably a homology of 90% or more, more preferably a homology of 95% or more, most preferably a homology of 99% or more. Further, by combining an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in the heavy chain or light

chain amino acid sequence, it is also possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies. The number of amino acid residues to be substituted, deleted, or added is generally 10 or fewer, preferably 5 to 6 or fewer, more preferably 2 to 3 or fewer, most preferably 1.

The homology between two amino acid sequences can be determined using the Blast algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25: 3389-3402) with default parameters. The Blast algorithm can also be used through the Internet by accessing the site www.ncbi.nlm.nih.gov/blast. Incidentally, two types of percentage values of identity (or identities) and positivity (or positivities) are calculated by the Blast algorithm. The former is a value when amino acid residues match each other in two amino acid sequences for which a degree of homology is to be determined, and the latter is a value obtained by also considering amino acid residues having a similar chemical structure. In this specification, the value of the identity when amino acid residues match each other is used as the homology value.

Incidentally, in the heavy chain amino acid sequence

represented by SEQ ID NO: 34, 36, 38, 40, 42, 44, 46, 48, 50, or 70 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 19 is a signal sequence, an amino acid sequence consisting of amino acid residues 20 to 141 is a variable region, and an amino acid sequence consisting of amino acid residues 142 to 471 is a constant region. Further, in the light chain amino acid sequence represented by SEQ ID NO: 28, 30, 32, 52, 58, 62, or 66 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 20 is a signal sequence, an amino acid sequence consisting of amino acid residues 21 to 134 is a variable region, and an amino acid sequence consisting of amino acid residues 135 to 239 is a constant region.

The heavy chain amino acid sequence represented by SEQ ID NO: 34, 36, 38, 40, 42, 44, 46, 48, 50, or 70 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 33, 35, 37, 39, 41, 43, 45, 47, 49, or 69 in the Sequence Listing. In each of the above nucleotide sequences, a nucleotide sequence consisting of nucleotides 1 to 57 encodes the heavy chain signal sequence of the antibody, a nucleotide sequence consisting of nucleotides 58 to 423 encodes the heavy chain variable region of the antibody, and a nucleotide sequence consisting of nucleotides 424 to 1413 encodes the heavy chain constant region of the antibody.

The light chain amino acid sequence represented by SEQ

ID NO: 28, 30, 32, 52, 58, 62, or 66 in the Sequence Listing is encoded by a nucleotide sequence represented by SEQ ID NO: 27, 29, 31, 51, 57, 61, or 65 in the Sequence Listing. In each of the above nucleotide sequences, a nucleotide sequence consisting of nucleotides 1 to 60 encodes the light chain signal sequence of the antibody, a nucleotide sequence consisting of nucleotides 61 to 402 encodes the light chain variable region of the antibody, and a nucleotide sequence consisting of nucleotides 403 to 717 encodes the light chain constant region of the antibody.

The homology between any of these nucleotide sequences and a nucleotide sequence of another antibody can also be determined using the Blast algorithm.

Further, the antibody of the invention includes a human antibody which binds to the same epitope as the B273 antibody. A human anti-DR5 antibody refers to a human antibody only having a gene sequence of an antibody derived from a human chromosome. A human anti-DR5 antibody can be obtained by a method using a human antibody-producing mouse having a human chromosome fragment containing heavy and light chain genes of a human antibody (see Tomizuka, K. et al., Nature Genetics (1997) 16, pp. 133-143; Kuroiwa, Y. et al., Nucl. Acids Res. (1998) 26, pp. 3447-3448; Yoshida, H. et al., Animal Cell Technology: Basic and Applied Aspects vol. 10, pp. 69-73 (Kitagawa, Y., Matsuda, T. and Iijima, S. eds.), Kluwer Academic Publishers,

1999; Tomizuka, K. et al., Proc. Natl. Acad. Sci. USA (2000) 97, pp. 722-727, etc.).

Such a human antibody-producing mouse can be created specifically as follows. A genetically modified animal in which endogenous immunoglobulin heavy and light chain gene loci have been disrupted, and instead, human immunoglobulin heavy and light chain gene loci have been introduced via a yeast artificial chromosome (YAC) vector or the like is created by producing a knockout animal and a transgenic animal and mating these animals.

Further, according to a genetic engineering technique, by using cDNAs encoding such a heavy chain and a light chain of a human antibody, respectively, and preferably a vector containing the cDNAs, eukaryotic cells are transformed, and a transformant which produces a recombinant human monoclonal antibody is cultured, whereby the antibody can also be obtained from the culture supernatant.

Here, as the host, for example, eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes or myeloma cells can be used.

Further, a method of obtaining a phage display-derived human antibody screened from a human antibody library (see Wormstone, I. M. et al., Investigative Ophthalmology & Visual Science. (2002) 43 (7), pp. 2301-2308; Carmen, S. et al., Briefings in Functional Genomics and Proteomics (2002), 1 (2),

pp. 189-203; Siriwardena, D. et al., Ophthalmology (2002) 109 (3), pp. 427-431, etc.) is also known.

For example, a phage display method in which a variable region of a human antibody is expressed on the surface of a phage as a single-chain antibody (scFv), and a phage which binds to an antigen is selected (Nature Biotechnology (2005), 23, (9), pp. 1105-1116) can be used.

By analyzing the gene of the phage selected based on the binding to an antigen, a DNA sequence encoding the variable region of a human antibody which binds to an antigen can be determined.

If the DNA sequence of a scFv which binds to an antigen is determined, a human antibody can be obtained by preparing an expression vector having the sequence and introducing the vector into an appropriate host to express it (WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, WO 95/15388, Annu. Rev. Immunol. (1994) 12, pp. 433-455, Nature Biotechnology (2005) 23 (9), pp. 1105-1116).

If a newly produced human antibody binds to a partial peptide or a partial tertiary structure to which the B273 antibody binds, it can be determined that the human antibody and the B273 antibody bind to the same epitope. Further, by confirming that the human antibody competes with the B273 antibody for the binding to DR5 (that is, the human antibody inhibits the binding between the B273 antibody and DR5), it

can be determined that the human antibody and the B273 antibody bind to the same epitope even if the specific epitope sequence or structure has not been determined. When it is confirmed that the human antibody and the B273 antibody bind to the same epitope, the human antibody is strongly expected to have a cytotoxic activity equivalent to that of B273.

Further, as shown in Example 4, it is possible to specify an amino acid residue on the side of DR5 which lies adjacent to a Fab fragment of an antibody from the data of X-ray diffraction of a complex between the Fab fragment and DR5. Specifically, in the case where a Fab fragment derived from an arbitrary antibody lies adjacent to a glycine residue at position 26, an isoleucine residue at position 34, a glutamic acid residue at position 36, an aspartic acid residue at position 37, a glycine residue at position 38, an aspartic acid residue at position 56, a leucine residue at position 57, a leucine residue at position 58, a phenylalanine residue at position 59, a leucine residue at position 61, and an arginine residue at position 62 of an amino acid sequence represented by SEQ ID NO: 23 in the Sequence Listing, at a distance of 4 Å or less, it can be determined that the antibody binds to the same epitope as B273.

The chimeric antibodies, humanized antibodies, or human antibodies obtained by the above-described method are evaluated for the property of binding to an antigen by a method

shown in Example 3 or the like, and a preferred antibody can be selected. As one example of another index for use in the comparison of the properties of antibodies, the stability of antibodies can be exemplified. The differential scanning calorimetry (DSC) shown in Example 10 is a device capable of quickly and accurately measuring a thermal denaturation midpoint temperature (Tm) to be used as a favorable index of the relative conformational stability of proteins. By measuring the Tm values using DSC and comparing the values, the difference in thermal stability can be compared. It is known that the storage stability of antibodies shows some correlation with the thermal stability of antibodies (Lori Burton, et. al., Pharmaceutical Development and Technology (2007) 12, pp. 265-273), and a preferred antibody can be selected by using thermal stability as an index. Examples of other indices for selecting antibodies include the following factors: the yield in an appropriate host cell is high and the aggregability in an aqueous solution is low. For example, an antibody which shows the highest yield does not always show the highest thermal stability, and therefore, it is necessary to select an antibody most suitable for the administration to humans by making a comprehensive evaluation based on the above-described indices.

Further, a method in which the full-length heavy and light chain sequences of an antibody are connected using an

appropriate linker, whereby a single-chain immunoglobulin is obtained is also known (Lee, H-S, et. al., Molecular Immunology (1999) 36, pp. 61-71; Shirrmann, T. et. al., mAbs (2010), 2, (1) pp. 1-4). By dimerizing such a single-chain immunoglobulin, the resulting dimer can have a structure and an activity similar to those of an antibody which is a tetramer itself. Further, the antibody of the invention may be an antibody which has a single heavy chain variable region and does not have a light chain sequence. Such an antibody is called a single domain antibody (sdAb) or a nanobody, and in fact, such an antibody is observed in camels and llamas and has been reported to have an antigen-binding affinity (Muyldemans S. et. al., Protein Eng. (1994) 7 (9), 1129-35, Hamers-Casterman C. et. al., Nature (1993) 363 (6428) 446-8). The above-described antibodies are included in the antibody according to the invention.

Further, by controlling glycosylation in which a glycan is bound to the antibody of the invention, it is possible to enhance antibody-dependent cytotoxic activity. As the technique for controlling the glycosylation of antibodies, WO 99/54342, WO 00/61739, WO 02/31140, etc. are known. However, it is not limited thereto.

In the case where an antibody is produced by first isolating an antibody gene and then introducing the gene into an appropriate host, a combination of an appropriate host and

an appropriate expression vector can be used. Specific examples of the antibody gene include a combination of a gene encoding a heavy chain sequence of an antibody described in this specification and a gene encoding a light chain sequence thereof. When a host cell is transformed, it is possible to insert the heavy chain sequence gene and the light chain sequence gene into the same expression vector, and also into different expression vectors separately. In cases where eukaryotic cells are used as the host, animal cells, plant cells, and eukaryotic microorganisms can be used. As the animal cells, (1) mammalian cells, for example, dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61) can be exemplified. Further, in the case where prokaryotic cells are used, for example, Escherichia coli and Bacillus subtilis can be exemplified. By introducing a target antibody gene into these cells through transformation, and culturing the thus transformed cells in vitro, the antibody can be obtained. In the above-described culture method, the yield may sometimes vary depending on the sequence of the antibody, and therefore, it is possible to select an antibody which is easily produced as a pharmaceutical by using the yield as an index among the

antibodies having comparable binding activity.

There is no limitation on isotype of the antibody of the invention, and examples thereof include IgG (IgG1, IgG2, IgG3, IgG4), IgM, IgA (IgA1, IgA2), IgD, and IgE, and preferred examples thereof include IgG and IgM, and further more preferred examples thereof include IgG1 and IgG2.

Further, the antibody of the invention may be a functional fragment of the antibody having an antigen-binding site of the antibody or a modified fragment thereof. The fragment of the antibody can be obtained by treating the antibody with a protease such as papain or pepsin, or modifying the antibody gene according to a genetic engineering technique and expressing the modified gene in suitable cultured cells. Among these antibody fragments, a fragment having all or part of the functions of the full-length molecule of the antibody can be called a functional fragment of the antibody. As the functions of the antibody, generally an antigen-binding activity, an activity of neutralizing the activity of an antigen, an activity of enhancing the activity of an antigen, an antibody-dependent cytotoxic activity, a complement-dependent cytotoxic activity, and a complement-dependent cellular cytotoxic activity can be exemplified. The function of the functional fragment of the antibody according to the invention is the activity of binding to DR5, preferably the activity of inducing apoptosis in cells,

more preferably the cytotoxic activity through the induction of apoptosis in cancer cells. However, the antibody of the invention may have antibody-dependent cytotoxic activity, complement-dependent cytotoxic activity, and/or complement-dependent cellular cytotoxic activity as well as the activity of inducing apoptosis in cells.

Examples of the fragment of the antibody include Fab, F(ab')2, Fv, single-chain Fv (scFv) in which Fv molecules of the heavy chain and the light chain are connected via an appropriate linker, a diabody (diabodies), a linear antibody, and a polyspecific antibody composed of the antibody fragment. Further, Fab' which is a monovalent fragment in a variable region of an antibody obtained by treating F(ab')2 under reducing conditions is also included in the fragment of the antibody.

Further, the antibody of the invention may be a polyspecific antibody with specificity for at least two different antigens. In general, such a molecule binds to two antigens (that is, bispecific antibody), however, the term "polyspecific antibody" as used herein includes an antibody having specificity for two or more (for example, three) antigens.

The polyspecific antibody of the invention may be a full-length antibody or a fragment of such an antibody (for example, a F(ab')2 bispecific antibody). The bispecific

antibody can be produced by connecting the heavy and light chains (HL pairs) of two types of antibodies, or can also be produced by fusing hybridomas which produce different monoclonal antibodies to prepare bispecific antibody-producing fused cells (Millstein et al., Nature (1983) 305, pp. 537-539).

The antibody of the invention may be a single-chain antibody (also referred to as scFv). The single-chain antibody can be obtained by connecting the heavy chain variable region and the light chain variable region of the antibody via a polypeptide linker (Pluckthun, The Pharmacology of Monoclonal Antibodies, 113 (edited by Rosenburg and Moore), Springer Verlag, New York, pp. 269-315 (1994), Nature Biotechnology (2005), 23, pp. 1126-1136). Further, a BiscFv fragment produced by connecting two scFv molecules via a polypeptide linker can also be used as the bispecific antibody.

A method of producing a single-chain antibody is known in this technical field (see, for example, US patent Nos. 4,946,778, 5,260,203, 5,091,513, 5,455,030, etc.). In this scFv, the heavy chain variable region and the light chain variable region are connected via a linker which does not form a conjugate, preferably via a polypeptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. USA (1988), 85, pp. 5879-5883). In the scFv, the heavy chain variable region and the light chain variable region may be derived from the same antibody or

different antibodies.

As the polypeptide linker to be used for connecting the variable regions, a given single-chain peptide composed of 12 to 19 residues is used, for example.

DNA encoding scFv can be obtained by performing amplification using a DNA encoding the entire amino acid sequence or a desired partial amino acid sequence of a DNA selected from a DNA encoding the heavy chain or the heavy chain variable region of the above-described antibody and a DNA encoding the light chain or the light chain variable region thereof as a template by a PCR method using a primer pair that defines both ends thereof, and further performing amplification by combining a DNA encoding a polypeptide linker portion and a primer pair that defines both ends thereof so as to connect the both ends to the heavy chain and the light chain, respectively.

Further, once DNA encoding a scFv is produced, an expression vector containing the same and a host transformed by the expression vector can be obtained according to a common procedure. Further, by using the resulting host, scFv can be obtained according to a common procedure. An antibody fragment thereof can be produced in a host by obtaining a gene and expressing the gene in the same manner as described above.

The antibody of the invention may be multimerized to increase its affinity for an antigen. The antibody to be

multimerized may be one type of antibody or a plurality of antibodies which recognize a plurality of epitopes of the same antigen. As a method of multimerization of the antibody, binding of the IgG CH3 domain to two scFv molecules, binding to streptavidin, introduction of a helix-turn-helix motif, and the like can be exemplified.

The antibody of the invention may be a polyclonal antibody which is a mixture of plural types of anti-DR5 antibodies having different amino acid sequences. As one example of the polyclonal antibody, a mixture of plural types of antibodies having different CDR can be exemplified. As such a polyclonal antibody, antibodies obtained by culturing a mixture of cells which produce different antibodies and then purifying the antibodies from the resulting culture can be used (see WO 2004/061104).

As a modified antibody, an antibody bound to any of various types of molecules such as polyethylene glycol (PEG) can also be used.

Further, the antibody of the invention may be in the form of a conjugate formed between any of these antibodies and another medicinal agent (immunoconjugate). Examples of such an antibody include one in which the antibody is conjugated to a radioactive material or a compound having a pharmacological action (Nature Biotechnology (2005) 23, pp. 1137-1146).

The obtained antibody can be purified to homogeneity. The separation and purification of the antibody can be performed employing a conventional protein separation and purification method. For example, the antibody can be separated and purified by appropriately selecting and combining column chromatography, filter filtration, ultrafiltration, salt precipitation, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing electrophoresis, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the method is not limited thereto.

Examples of such chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and adsorption chromatography.

Such chromatography can be performed employing liquid chromatography such as HPLC or FPLC.

As a column to be used in affinity chromatography, a Protein A column and a Protein G column can be exemplified.

For example, as a column using a Protein A column, Hyper D, POROS, Sepharose FF (Pharmacia) and the like can be exemplified. Further, by using a carrier having an antigen

immobilized thereon, the antibody can also be purified utilizing the binding property of the antibody to the antigen.

(4) Specific examples of other anti-DR5 antibodies

Anti-DR5 antibodies which induce apoptosis in DR5-expressing cells are described in, for example, WO 98/51793, WO 2001/83560, WO 2002/94880, WO 2003/54216, WO 2006/83971, and WO 2007/22157. Further, anti-DR5 antibodies called tigatuzumab (CS-1008), lexatumumab (HGS-ETR2), HGS-TR2J, drozitumab (APOMAB), conatumumab (AMG-655), and LBY135 are still in clinical trials or were in clinical trials in the past. The anti-DR5 antibodies which were still in clinical trials on the date when this application was filed are tigatuzumab, lexatumumab, and conatumumab. The novel anti-DR5 antibodies described in this specification have a superior in vitro and/or in vivo antitumor activity as compared with the above-described tigatuzumab, lexatumumab, conatumumab, and drozitumab.

3. Pharmaceutical containing anti-DR5 antibody

The antibodies obtained by the method described in the above item “2. Production of anti-DR5 antibody” can be used as a pharmaceutical, particularly a therapeutic and/or preventive agent for cancer since the antibodies each function as an agonist for an apoptosis-related receptor, DR5, in vivo and induce apoptosis in cancer cells via the receptor to exhibit a cytotoxic activity.

The cytocidal activity exhibited by an antibody in vitro can be determined by measuring their activity in inhibiting the proliferation of cells which overexpress an apoptosis-related receptor.

For example, a cancer cell line which overexpresses DR5 is cultured, an antibody is added to the culture system at different concentrations, and inhibitory activity against focus formation, colony formation, and spheroid proliferation can be measured.

The in vivo therapeutic effect of an antibody on cancer using experimental animals can be determined by, for example, measuring a change in cancer cells by administering the antibody to nude mice implanted with a tumor cell line which overexpresses DR5.

Examples of the type of cancer include lung cancer, prostate cancer, thyroid cancer, stomach cancer, liver cancer, ovarian cancer, colon cancer, breast cancer, pancreatic cancer, kidney cancer, uterine cancer including endometrial cancer, melanocarcinoma including melanoma, fibrosarcoma, glioblastoma, and blood cell cancer (such as leukemia and lymphoma), however, the type of cancer is not limited thereto as long as the cancer cell to be treated expresses DR5.

Further, it is known that an antibody against DR5 induces apoptosis in inflammatory cells (J. Clin. Invest. 1996, 98 (2), 271-278; Int. Immunol. 1996, 8 (10), 1595-1602). Therefore,

the antibody of the invention can be also used as a therapeutic agent for an autoimmune disease or an inflammatory disease. Examples of the autoimmune disease or inflammatory disease include systemic lupus erythematosus, Hashimoto's disease, rheumatoid arthritis, a graft-versus-host disease, Sjogren's syndrome, pernicious anemia, Addison's disease, scleroderma, Goodpasture's syndrome, Crohn's disease, autoimmune hemolytic anemia, sterility, myasthenia gravis, multiple sclerosis, Basedow's disease, thrombopenia purpura, insulin-dependent diabetes mellitus, allergy, asthma, an atopic disease, arteriosclerosis, myocarditis, cardiomyopathy, glomerulonephritis, aplastic anemia, and rejection after organ transplantation.

A substance to be used in a preparation acceptable in the pharmaceutical composition according to the invention is preferably non-toxic to a person to whom the pharmaceutical composition is to be administered in terms of the dose and concentration.

The pharmaceutical composition of the invention can contain a substance for pharmaceutical use which is capable of changing or maintaining the pH, osmotic pressure, viscosity, transparency, color, isotonicity, aseptic condition, stability, solubility, release rate, absorption rate, and permeability thereof. Examples of such a substance for pharmaceutical use include, but are not limited to, amino acids

such as glycine, alanine, glutamine, asparagine, arginine, and lysine; antimicrobial agents; antioxidants such as ascorbic acid, sodium sulfate, and sodium hydrogen sulfite; buffers such as phosphate, citrate, borate buffers, sodium hydrogen carbonate, and Tris-HCl solutions; fillers such as mannitol and glycine; chelating agents such as ethylenediamine tetraacetate (EDTA); complexing agents such as caffeine, polyvinylpyrrolidine, β-cyclodextrin, and hydroxypropyl-β-cyclodextrin; expanders such as glucose, mannose, and dextrin; other carbohydrates such as monosaccharides and disaccharides; coloring agents; flavors; diluents; emulsifying agents; hydrophilic polymers such as polyvinylpyrrolidine; preservatives such as low molecular weight polypeptides, salt forming counter ions, benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, and hydrogen peroxide; solvents such as glycerin, propylene glycol, and polyethylene glycol; sugar alcohols such as mannitol and sorbitol; suspending agents; surfactants such as sorbitan ester, polysorbates including polysorbate 20 and polysorbate 80, Triton, tromethamine, lecithin, and cholesterol; stability enhancing agents such as sucrose and sorbitol; elasticity enhancing agents such as sodium chloride, potassium chloride, and mannitol and sorbitol; transport agents; excipients; and/or pharmaceutical adjuvants. The

amount of these substances for pharmaceutical use is preferably from 0.01 to 100 times, particularly preferably from 0.1 to 10 times the weight of the anti-DR5 antibody. Those skilled in the art can appropriately determine a preferred formulation of the pharmaceutical composition in a preparation depending on the disease to which the composition is applied, the route of administration to be applied, or the like.

The excipient or carrier in the pharmaceutical composition may be in the form of a liquid or a solid. An appropriate excipient or carrier may be injectable water, physiological saline, an artificial cerebral spinal fluid, or other substance commonly used for parenteral administration. Further, neutral physiological saline or physiological saline containing serum albumin can also be used as a carrier. The pharmaceutical composition may contain a Tris buffer of pH 7.0 to 8.5, an acetate buffer of pH 4.0 to 5.5, or a citrate buffer of pH 3.0 to 6.2. Further, such a buffer may be supplemented with sorbitol or another compound.

Examples of the pharmaceutical composition of the invention include a pharmaceutical composition containing the anti-DR5 antibody and a pharmaceutical composition containing the anti-DR5 antibody and at least one therapeutic agent for cancer. The pharmaceutical composition of the invention is prepared in the form of a lyophilized product or a liquid as a medicinal agent having a selected composition and a required

purity. The pharmaceutical composition containing the anti-DR5 antibody and the pharmaceutical composition containing the anti-DR5 antibody and at least one therapeutic agent for cancer can also be formed into a lyophilized product using an appropriate excipient such as sucrose.

In the above-described pharmaceutical composition, the therapeutic agent for cancer to be incorporated along with the anti-DR5 antibody may be administered simultaneously with, separately from, or sequentially with the anti-DR5 antibody, or the therapeutic agent and the anti-DR5 antibody may be administered at different dosage intervals. Examples of such a therapeutic agent for cancer include abraxane, carboplatin, cisplatin, gemcitabine, irinotecan (CPT-11), paclitaxel, pemetrexed, sorafenib, vinblastin, 5-FU, and medicinal agents described in WO 2003/038043, however, the agent is not limited thereto as long as the agent is a medicinal agent having an antitumor activity.

The pharmaceutical composition of the invention can be prepared for parenteral administration or for gastrointestinal absorption through oral administration. The composition and concentration of a preparation can be determined depending on the administration method. The higher the affinity of the anti-DR5 antibody contained in the pharmaceutical composition of the invention is for DR5, that is, the lower the dissociation constant (Kd value) thereof is

for DR5, the more the anti-DR5 antibody can exhibit its drug efficacy even when decreasing the dose for humans. Hence, the dose of the pharmaceutical composition of the invention for humans can also be determined based on this fact. As for the dose, in the case where a human anti-DR5 antibody is administered to humans, the antibody may be administered at a dose of from about 0.1 to 100 mg/kg once per one to 180 days.

Examples of the dosage form of the pharmaceutical composition of the invention include injections including infusions, suppositories, transnasal agents, sublingual agents, and percutaneous absorbents.

Hereinafter, the invention will be more specifically described with reference to the Examples, however, the invention is not limited thereto. Note that the respective operations regarding gene manipulation in the following Examples were performed according to the methods described in "Molecular Cloning" (written by Sambrook, J., Fritsch, E. F. and Maniatis, T., published by Cold Spring Harbor Laboratory Press in 1989), or in the case of using commercially available reagents or kits, they are used according to the protocols attached thereto unless otherwise stated.

[Example 1] Production of mouse antibody B273

1)-1 Production of human DR5 protein (human DR5 extracellular domain/human Fc fusion protein)

1)-1-1 Production of human DR5 extracellular domain expression vector

A vector expressing human DR5 protein (isoform 2: NP_671716) was constructed by inserting a gene, in which a human DR5 extracellular domain was fused to a human IgG1/Fc region, downstream of the CMV promoter.

1)-1-2 Production of human DR5 protein

The introduction of the expression vector into 293 FreeStyle cells and the collection of the culture supernatant were performed by Invitrogen Corporation (currently Life Technologies Japan Ltd.).

1)-1-3 Purification of human DR5 protein

The culture supernatant obtained in the above b) was purified using Protein A affinity column chromatography. 5 L of the culture supernatant was applied to “HiTrap Protein AFF” (GE Healthcare Bio-Sciences Co., Ltd., Cat. No. 17-5079-01) equilibrated with PBS, followed by washing with PBS. Subsequently, a 2 M arginine solution (pH 4.0) was added to the column, and a fraction containing the human DR5 protein was collected. The fraction was added to a centrifugal filter device (Amicon Ultra-4, fractional molecular weight: 10 K, Millipore Co., Ltd.), and liquid replacement with PBS and condensation were performed. The final volume was made up to

6 ml, which was used as a purified sample (rDR5-hFc). The quantitative determination of the purified product of protein was performed using “Micro BCA Protein Assay Kit” (PIERCE #23235). As a reference standard, the “Albumin Standard” contained in the kit was used.

1)-2 Immunization

BALB/cAJcl mice (CLEA Japan, Inc.) at 5 to 6 weeks of age were used. On day 0, a mixture of 50 μg of rDR5-hFc prepared in 1)-1-3 and Freund’s complete adjuvant (manufactured by Wako Pure Chemical Industries, Ltd.) (at a volume ratio of 1:1) was subcutaneously administered in the vicinity of the neck of each mouse. On days 14 and 28, a mixture of 50 μg of rDR5-hFc and Freund’s incomplete adjuvant (manufactured by Wako Pure Chemical Industries, Ltd.) (at a volume ratio of 1:1) was subcutaneously administered in the dorsal region of each mouse. On day 42, 50 μg of rDR5-hFc was administered into the abdominal cavity of each mouse, and on day 45, the spleen was excised from each mouse and used for the production of hybridomas.

1)-3 Production of hybridomas

Spleen cells and mouse myeloma P3X63Ag8U.1 cells were subjected to cell fusion using PEG 4000 (manufactured by Immuno-biological Laboratories Co., Ltd.), and the resulting fused cells were diluted with ClonaCell-HY Selection Medium

D (manufactured by StemCell Technologies, Inc., #03804) and cultured. Then, the hybridoma colonies formed were collected, whereby monoclonal hybridomas were produced. The collected hybridoma colonies were separately cultured, and by using the obtained culture supernatant of each hybridoma, an anti-DR5 antibody-producing hybridoma was screened.

1)-4 Screening of antibody by cell-ELISA method

1)-4-1 Construction of human DR5 mutant expression vector (pcDNA3.1-DR5M)

A cDNA encoding the human DR5 protein (isoform 2: NP_671716) was cloned into a pcDNA3.1(+) vector, and a death domain-modified expression vector pcDNA3.1-DR5M which was designed so as to express a protein in which the amino acid L at position 334 in the death domain is substituted with D was constructed.

1)-4-2 Preparation of antigen gene-expressing cells

HEK 293 cells were prepared at 7.5 x 10^5 cells/ml in DMEM medium containing 10% FBS. Then, the HEK 293 cells were transfected with the death domain-modified DR5 expression vector pcDNA3.1-DR5M or pcDNA3.1-mock serving as a control using Lipofectamine 2000 (manufactured by Life Technologies Japan Ltd.), and each cell suspension was dispensed at 50 μl/well in a 96-well half area microplate (manufactured by

Corning Incorporated). The cells were cultured overnight in DMEM medium containing 10% FBS under the conditions of 37°C and 5% CO_2. The thus obtained transfected cells in an adherent state were used as such in a cell-ELISA.

1)-4-3 Cell-ELISA

After the supernatant was removed from the culture of the HEK 293 cells transfected with the expression vector prepared in 1)-4-2, the hybridoma culture supernatant was added to each of the HEK 293 cells transfected with pcDNA3.1-DR5M and the HEK 293 cells transfected with pcDNA3.1-mock, and the plate was left to stand at 4°C for 1 hour. After the cells in each well were washed once with PBS containing 5% FBS, goat anti-mouse IgG, peroxidase conjugated (manufactured by Chemicon Co., Ltd., #AP181P) diluted to 500-fold with PBS containing 5% FBS was added to each well, and the plate was left to stand at 4°C for 1 hour. After the cells in each well were washed 5 times with PBS containing 5% FBS, an OPD color developing solution (o-phenylenediamine dihydrochloride (manufactured by Wako Pure Chemical Industries, Ltd.) and H_2O_2 were dissolved at 0.4 mg/ml and 0.6% (v/v), respectively, in a solution for dissolving OPD (0.05 M trisodium citrate and 0.1 M disodium hydrogen phosphate dodecahydrate, pH 4.5)) was added at 25 μl/well. A color development reaction was allowed to proceed while sometimes stirring the reaction mixture, and

the color development reaction was stopped by adding 1 M HCl at 25 μl/well. Thereafter, an absorbance at 490 nm was measured using a plate reader (ARVO, manufactured by Perkin Elmer, Inc.). In order to select a hybridoma which produces an antibody that specifically binds to DR5 expressed on the cell membrane, a hybridoma which produces a culture supernatant showing a higher absorbance in the HEK 293 cells transfected with the pcDNA3.1-DR5M expression vector as compared with the HEK 293 cells transfected with the pcDNA3.1-mock (control) was selected to be positive for the production of an anti-DR5 antibody.

1)-5 Screening of antibody by flow cytometric method

1)-5-1 Preparation of antigen gene-expressing cells

293T cells were seeded at 5 x 10^4 cells/cm^2 in a 225-cm^2 flask (manufactured by Sumitomo Bakelite Co., Ltd.), and cultured overnight in DMEM medium containing 10% FBS under the conditions of 37°C and 5% CO_2. On the next day, the 293T cells were transfected with pcDNA3.1-DR5M or pcDNA3.1-mock serving as a control using Lipofectamine 2000, and further cultured overnight under the conditions of 37°C and 5% CO_2. On the next day, the 293T cells transfected with the expression vector were treated with TrypLE Express (manufactured by Life Technologies Japan Ltd.). Then, the cells were washed with DMEM containing 10% FBS, and thereafter suspended in PBS containing 5% FBS.

The thus obtained cell suspension was used in a flow cytometric analysis.

1)-5-2 Flow cytometric analysis

The 293T cell suspension prepared in 1)-5-1 was centrifuged and the supernatant was removed. Then, the hybridoma culture supernatant was added to each of the 293T cells transfected with pcDNA3.1-DR5M and the 293T cells transfected with pcDNA3.1-mock to suspend the cells, and the cells were left to stand at 4°C for 1 hour. After the cells were washed twice with PBS containing 5% FBS, fluorescein-conjugated goat IgG fraction to mouse IgG (whole molecule) (manufactured by Cappel Co., Ltd., #55493) diluted 1000-fold with PBS containing 5% FBS was added thereto to suspend the cells, and the cells were left to stand at 4°C for 1 hour. After the cells were washed 3 times with PBS containing 5% FBS, the cells were resuspended in PBS containing 5% FBS supplemented with 2 μg/ml 7-aminoactinomycin D (manufactured by Invitrogen (Molecular Probes) Corporation), and the detection was performed using a flow cytometer (FC500, Beckman Coulter, Inc.). The data was analyzed using Flowjo (Tree Star, Inc.). 7-Aminoactinomycin D-positive dead cells were excluded using a gate. Then, the FITC fluorescence intensity histograms of viable cells were created. A hybridoma which produced a sample that gave a higher fluorescence intensity

in the fluorescence intensity histogram of the 293T cells transfected with pcDNA3.1-DR5M than in the fluorescence intensity histogram of the 293T cells transfected with pcDNA3.1-mock serving as the control was selected to be positive for the production of an anti-DR5 antibody.

1)-6 Screening in terms of cytocidal effect

By using the culture supernatants of the hybridomas selected to be positive for the production of an anti-DR5 antibody in 1)-4 and 1)-5, a cell death-inducing effect on a human T-lymphoma cell line Jurkat was confirmed. AffiniPure goat anti-mouse IgG Fc specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #115-005-071) prepared at 50 μg/ml with 5 mM Tris-HCl (pH 8.5) was dispensed at 25 μL/well in a 96-well half area microplate (manufactured by Corning Incorporated), and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, a hybridoma culture supernatant was added to each well and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, Jurkat cells prepared at 4.0×10^4 cells/ml in RPMI 1640 medium containing 10% FBS were added at 25 μl/well and cultured under the conditions of 37°C and 5% CO_2 for 20 hours. The cytocidal effect of the anti-DR5 monoclonal antibody present in the hybridoma culture supernatant was evaluated by quantitatively determining the amount of ATP derived from

viable cells using a CellTiter-Glo luminescent cell viability assay kit (manufactured by Promega Corporation, #G7571). As a result, hybridomas which produce 5 types of monoclonal antibodies (B086, B139, B192, B273, and B467), each of which exhibited a decrease in the amount of ATP by 80% or more as compared with the case of adding the medium for culturing the hybridoma, were established.

1)-7 Isotype determination of monoclonal antibody

The isotypes of the monoclonal antibodies were determined using Mouse monoclonal isotyping kit (manufactured by AbD Serotec, Inc.). As a result, the isotype of B086, B139, B192, B273, and B467 was confirmed to be IgG1, κ chain.

1)-8 Preparation of monoclonal antibody

The monoclonal antibody was purified from the ascites of a mouse implanted with a hybridoma (hereinafter, referred to as a “starting material for antibody purification”).

The mouse ascites was prepared as follows. First, BALB/cAJcl-nu/nu (Japan SLC, Inc.) mice of 7 to 8 weeks of age were treated with pristane (manufactured by Sigma Co., Ltd.), and after about 3 weeks, a hybridoma washed with physiological saline was implanted into the abdominal cavity at 1×10^7 cells per mouse. After 1 to 2 weeks, the ascites accumulated in the abdominal cavity was collected and sterilized through a 0.22-μm

mesh filter, and the resulting material was used as a starting material for antibody purification.

The antibody was purified by Hitrap MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Co., Ltd.). That is, the starting material for antibody purification was added to a column, and the column was washed with PBS, and thereafter, elution was performed with 2 M Arginine-HCl pH 4.0. After the eluted antibody solution was neutralized, the buffer was replaced with PBS. The concentration of the antibody was obtained by eluting the antibody bound to POROS G 20 μm Column PEEK, 4.6 mm × 50 mm, 0.83 ml (Applied Biosystems, Inc.) and measuring the absorbance (O.D. 280 nm) of the eluate. Specifically, an antibody sample diluted with PBS was added to POROS G 20 μm equilibrated with an equilibrating buffer (30.6 mM sodium dihydrogen phosphate dodecahydrate, 19.5 mM monopotassium phosphate, 0.15 M NaCl, pH 7.0). Then, the column was washed with the equilibrating buffer, and the antibody bound to the column was then eluted with an eluent (0.1% (v/v) HCl, 0.15 M NaCl). The peak area of the absorbance (O.D. 280 nm) of the eluate was measured, and the concentration was calculated according to the following equation: Concentration of antibody sample (mg/ml) = (Peak area of antibody sample) / (Peak area of reference standard (human IgG1)) × Concentration of reference standard (mg/ml) × Dilution factor of sample. Moreover, the concentration of endotoxin

contained in the obtained antibody was measured using Limulus ES-II Single Test Wako (Wako Pure Chemical Industries, Ltd., 295-51030 containing control standard endotoxin) and a toxinometer (Wako Pure Chemical Industries, Ltd., ET-301 or ET-5000) and was confirmed to be 1 EU/mg or less. The resulting antibody was used in the subsequent experiment.

1)-9 In vitro cytocidal activity of mouse antibody B273 against human cancer cell lines

Each of a human T-lymphoma cell line Jurkat and a human glioblastoma cell line U-87MG was prepared at 4.4×10^4 cells/ml in RPMI 1640 medium containing 10% FBS or MEM (Minimum Essential Medium) medium containing 10% FBS and added to a white clear bottom 96-well microplate (manufactured by Corning Incorporated) at 45 μl/well, and cultured overnight under the conditions of 37°C and 5% CO_2. The mouse B273 antibody or the mouse IgG1 antibody (manufactured by R&D Systems, Inc.) was mixed with the same concentration of AffiniPure goat anti-mouse IgG Fc specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #115-005-071), and the resulting mixture was added at 5 μl/well such that the final concentration of the mouse B273 antibody or the mouse IgG1 antibody was 10,000 to 0.01 ng/ml, and the cells were cultured under the conditions of 37°C and 5% CO_2 for 24 hours. The amount of ATP derived from viable cells in each well was measured by a luminometer

(manufactured by Perkin Elmer, Inc.) using a CellTiter-Glo luminescent cell viability assay kit (manufactured by Promega Corporation, #G7571) according to the attached protocol. The cytocidal activity was evaluated by taking the value obtained from the well to which the medium was added in place of the antibody solution as 100% (Fig. 1). In each graph, the cell viability is expressed as a mean ± standard deviation (n = 3). As a result, it was found that the mouse B273 antibody exhibits a cytocidal effect on both cell lines in an antibody concentration-dependent manner.

[Example 2] Cloning of mouse antibody B273 gene and production of human chimeric antibody gene

2)-1 Cloning of mouse antibody B273 cDNA and determination of sequence

2)-1-1 Determination of N-terminal amino acid sequences of heavy and light chains of mouse antibody B273

In order to determine the N-terminal amino acid sequences of the heavy and light chains of the mouse antibody B273, the mouse antibody B273 purified in Example 1-8 was separated by SDS-PAGE. The protein in the gel was transferred from the gel, after separation, to a PVDF membrane (pore size: 0.45 μm, manufactured by Invitrogen Corporation). The membrane was washed with a washing buffer (25 mM NaCl, 10 mM sodium borate buffer pH 8.0), and thereafter stained by being immersed in

a dye solution (50% methanol, 20% acetic acid, 0.05% Coomassie brilliant blue) for 5 minutes, followed by destaining with 90% methanol. The portions of the band corresponding to the heavy chain (the band with smaller mobility) and the band corresponding to the light chain (the band with larger mobility) visualized on the PVDF membrane were excised, and an attempt was made to identify their respective N-terminal amino acid sequences by an automatic Edman method (see Edman et al. (1967) Eur. J. Biochem. 1, 80) using Procise (registered trademark) cLC Protein Sequencer Model 492cLC (Applied Biosystems, Inc.). As a result, the N-terminal amino acid sequence of the band corresponding to the heavy chain of the mouse antibody B273 was EVQLQQSGPELVKPG (SEQ ID NO: 1 in the Sequence Listing), and the N-terminal amino acid sequence of the band corresponding to the light chain of the mouse antibody B273 was DVVMTQTPLSLPVSLGDQAS (SEQ ID NO: 2 in the Sequence Listing).

2)-1-2 Preparation of mRNA from mouse antibody B273-producing hybridoma

In order to clone cDNAs encoding the heavy chain and the light chain of the mouse antibody B273, respectively, mRNA was prepared from the mouse antibody B273-producing hybridoma using Quick Prep mRNA Purification Kit (GE Healthcare Bio-Sciences Co., Ltd.).

2)-1-3 Cloning of mouse antibody B273 cDNA and determination of sequence

With reference to the findings that the isotypes of the heavy and light chains of the mouse antibody B273 are γ1 and κ found in Example 1-7, and the N-terminal amino acid sequences of the heavy and light chains determined in the above 2)-1-1, and the database of the amino acid sequences of antibodies prepared by Kabat et al. (see Kabat, E. A. et al., (1991) in Sequences of Proteins of Immunological Interest Vol. I and II, U.S. Department of Health and Human Services), several oligonucleotide primers hybridizing to the 5'-terminal region of an antibody gene coding region and the 3'-terminal region thereof containing a stop codon, respectively, were synthesized, and a cDNA encoding the heavy chain and a cDNA encoding the light chain were amplified using the mRNA prepared in 2)-1-2 and TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa Bio, Inc.). As a result, the cDNA encoding the heavy chain of the antibody and the cDNA encoding the light chain of the antibody could be amplified by the following primer sets.

A primer set for the heavy chain

5'-aagaattcatgggatggagctgtatc-3' (MH258E1F1: SEQ ID NO: 3 in the Sequence Listing)

5'-aagatatcttatttaccaggagagtgggagag-3' (G1EVR1: SEQ ID NO: 4 in the Sequence Listing)

A primer set for the light chain

5′-aagaattcatgaagttgcctgttagg-3′ (MK19EIF1: SEQ ID NO: 5 in the Sequence Listing)

5′-aagatatcttaacactcattcctgttgaagct-3′ (KEVR1: SEQ ID NO: 6 in the Sequence Listing)

Each of the cDNA encoding the heavy chain and the cDNA encoding the light chain amplified by PCR was cloned using pEF6/V5-His TOPO TA Expression Kit (Invitrogen Corporation), and each of the nucleotide sequences of the heavy chain and the light chain cloned was determined using a gene sequence analyzer ("ABI PRISM 3700 DNA Analyzer; Applied Biosystems" or "Applied Biosystems 3730xl Analyzer; Applied Biosystems"). In the sequencing reaction, GeneAmp 9700 (Applied Biosystems, Inc.) was used.

The determined nucleotide sequence of the cDNA encoding the heavy chain of the mouse antibody B273 is represented by SEQ ID NO: 7 in the Sequence Listing, and the amino acid sequence thereof is represented by SEQ ID NO: 8. The nucleotide sequence of the cDNA encoding the light chain of the mouse antibody B273 is represented by SEQ ID NO: 9 in the Sequence Listing, and the amino acid sequence thereof is represented by SEQ ID NO: 10 in the Sequence Listing. The sequences of SEQ ID NOS: 7 and 8 are shown in Fig. 28, and the sequences of SEQ ID NOS: 9 and 10 are shown in Fig. 29.

Further, the amino acid sequences of the heavy chain and

the light chain were analyzed by comparison using KabatMan (see PROTEINS: Structure, Function and Genetics, 25 (1996), 130-133), which is the database of the amino acid sequences of antibodies. As a result, it was found that in the heavy chain of the mouse antibody B273, the amino acid sequence represented by amino acid numbers 20 to 141 of SEQ ID NO: 8 in the Sequence Listing is a variable region. It was also found that in the light chain of the mouse antibody B273, the amino acid sequence represented by amino acid numbers 20 to 133 of SEQ ID NO: 10 in the Sequence Listing is a variable region.

2)-2 Production of chimeric antibody B273 expression vector

2)-2-1 Production of universal expression vectors pEF6KCL and pEF1FCCU

2)-2-1-1 Construction of chimeric and humanized light chain expression vector pEF6KCL

By performing PCR using the plasmid pEF6/V5-HisB (Invitrogen Corporation) as a template and also using the following primers, a DNA fragment from immediately downstream of BGHpA (Sequence Position: 2174) to SmaI (Sequence Position: 2958) (a DNA fragment containing f1 origin of replication and SV40 promoter and origin, hereinafter referred to as “fragment A”) was obtained.

5’-ccacgcgccctgtagcggcgcattaagc-3’ (primer EFF1: SEQ ID NO: 11 in the Sequence Listing)

5′-aaacccgggagctttttgcaaaagcctagg-3′ (primer EFsmaR: SEQ ID NO: 12 in the Sequence Listing)

The obtained fragment A and a DNA fragment (SEQ ID NO: 13, hereinafter referred to as “fragment B”) containing a DNA sequence encoding a human κ chain secretory signal, a human κ chain constant region, and a human poly-A additional signal were ligated to each other by overlap extension PCR. The thus obtained DNA fragment in which the fragment A and the fragment B were ligated to each other was digested with the restriction enzymes KpnI and SmaI, which was ligated to the plasmid pEF6/V5-HisB (Invitrogen Corporation) which was digested with the restriction enzymes KpnI and SmaI, whereby a chimeric and humanized light chain expression vector pEF6KCL having a signal sequence, a cloning site, a human κ chain constant region, and a human poly-A additional signal sequence downstream of the EF1 promoter was constructed.

2)-2-1-2 Construction of pEF1/KCL

A DNA fragment obtained by cleaving the pEF6KCL obtained by the above-described method with the restriction enzymes KpnI and SmaI was ligated to pEF1/myc-HisB (Invitrogen Corporation) which was digested with KpnI and SmaI, whereby a plasmid pEF1KCL was constructed.

2)-2-1-3 Construction of chimeric and humanized heavy chain

expression vector pEF1FCCU

A DNA fragment (SEQ ID NO: 14) containing a DNA sequence encoding amino acids of a signal sequence and a constant region of human IgG1 was digested with the restriction enzymes NheI and PmeI and was ligated to the plasmid pEF1KCL which was digested with NheI and PmeI, whereby a chimeric and humanized heavy chain expression vector pEF1FCCU having a signal sequence, a cloning site, a human heavy chain constant region, and a human poly-A additional signal sequence downstream of the EF1 promoter was constructed.

2)-2-2 Construction of B273 chimera-type light chain expression vector

By using the cDNA encoding the light chain of the mouse antibody B273 as a template and also using KOD-Plus- (TOYOBO, Co., Ltd.) and the following primer set, a region containing the cDNA encoding the light chain variable region was amplified. A DNA fragment obtained by cleaving the amplified product with the restriction enzymes NdeI and BsiWI was inserted into the universal chimeric and humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NdeI and BsiWI, whereby a B273 chimera-type light chain expression vector was constructed. The thus obtained expression vector was named “pEF6KCL/B273L”. The nucleotide sequence of the B273 chimera-type light chain is

represented by SEQ ID NO: 15 in the Sequence Listing, and the amino acid sequence thereof is represented by SEQ ID NO: 16. The sequences of SEQ ID NOS: 15 and 16 are shown in Fig. 30. Incidentally, the amino acid residue at position 134 in the amino acid sequence of the chimera-type light chain represented by SEQ ID NO: 16 in the Sequence Listing is located in the carboxyl terminus of the light chain variable region and corresponds to the alanine residue at position 133 in the amino acid sequence of the mouse antibody B273 light chain represented by SEQ ID NO: 10 in the Sequence Listing, however, in the amino acid sequence represented by SEQ ID NO: 16, the residue has already been substituted with a threonine residue derived from a human antibody light chain.

The primer set for the light chain:

5′-aaacatatggcgatgttgtgatgacccaaactccactctcc-3′ (B273LF: SEQ ID NO: 17 in the Sequence Listing)

5′-aaacgtacgtttgatttccagcttggtgcctccaccgaacg-3′ (B273LR: SEQ ID NO: 18 in the Sequence Listing)

2)-2-3 Construction of B273 chimera-type heavy chain expression vector

By using the cDNA encoding the heavy chain of the mouse antibody B273 as a template and also using KOD-Plus- (TOYOBO, Co., Ltd.) and the following primer set, a region containing the cDNA encoding the heavy chain variable region was amplified.

A DNA fragment obtained by cleaving the amplified product with the restriction enzyme BlpI was inserted into the universal chimeric and humanized antibody heavy chain expression vector (pEF1FCCU) at the site cleaved with the restriction enzyme BlpI, whereby a B273 chimera-type heavy chain expression vector was constructed. The thus obtained expression vector was named "pEF1FCCU/B273H". The nucleotide sequence of the B273 chimera-type heavy chain is represented by SEQ ID NO: 19 in the Sequence Listing, and the amino acid sequence thereof is represented by SEQ ID NO: 20. The sequences of SEQ ID NOS: 19 and 20 are shown in Fig. 31.

The primer set for the heavy chain:

5'-aaagctgagcgaggttcagctgcagcagtctggacctgagc-3' (B273HF: SEQ ID NO: 21 in the Sequence Listing)

5'-aaagctgagctgactgtgagagtggtgccttggccccagtag-3' (B273HR: SEQ ID NO: 22 in the Sequence Listing)

2)-3 Preparation of chimeric antibody B273

2)-3-1 Production of chimeric antibody B273

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen Corporation) in the logarithmic growth phase were seeded into 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen Corporation) and cultured for 1 hour by shaking at 90 rpm at 37°C in an 8% CO_2 incubator. 3.6 mg of polyethyleneimine (Polyscience #24765) was dissolved in 20 ml of Opti-Pro SFM

medium (Invitrogen Corporation). Subsequently, pEF1FCCU/B273H (0.4 mg) and pEF6KCL/B273L (0.8 mg) prepared with PureLink HiPure Plasmid Kit (Invitrogen Corporation) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the obtained expression vectors/Opti-Pro SFM mixed liquid was added to 20 ml of the obtained polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells, and culture shaking at 90 rpm was performed for 7 days at 37°C in an 8% CO_2 incubator. The resulting culture supernatant was filtered through a disposable capsule filter (Advantec #CCS-045-E1H).

A chimeric antibody B273 obtained by a combination of pEF1FCCU/B273H and pEF6KCL/B273L was named “cB273”.

2)-3-2 Purification of cB273

The culture supernatant obtained in the above 2)-3-1 was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap columns (volume:

1 ml) connected in series) equilibrated with PBS. After all culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0), and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the buffer was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to buffer replacement was applied to a ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1 hydroxyapatite column (volume: 2 ml)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), and the concentration of IgG was adjusted to 1.0 mg/ml or more, and

the thus obtained solution was used as a purified sample.

[Example 3] Measurement of activity of human chimeric B273 (cB273) antibody (in vitro)

3)-1 Study of selective binding property of cB273 antibody to human DR5 extracellular domain

The binding property of cB273 to extracellular domain proteins of human TRAIL R1 to R4 and mouse TRAIL R2 (manufactured by R&D Systems, Inc.) was studied by a direct ELISA method described below. First, each of the extracellular domain proteins of TRAIL Rs was diluted to 1 μg/ml with PBS, and the diluted solution was dispensed at 50 μl/well into an immunoplate (manufactured by Nunc, Inc., #442404), and the plate was left to stand overnight at 4°C, whereby the protein was adsorbed to the plate. On the next day, the liquid in each well was removed and each well was washed once with PBS. Thereafter, in order to suppress the non-specific adsorption of proteins, PBS containing 3% fetal bovine serum was dispensed at 200 μl/well, and the plate was left to stand at room temperature for 1.5 hours. The liquid in each well was removed and cB273 or soluble human TRAIL (manufactured by ALEXIS Corporation, #ALX-522-003) diluted with PBS containing 3% fetal bovine serum was added thereto at 50 μl/well. After the plate was left to stand at room temperature for 1.5 hours, PBS was added to each well, and then, the liquid in the well was

removed and the well was washed twice with PBS. Then, to the well to which the cB273 antibody was added, goat anti-Human IgG $F(ab')_2$ fragment specific, peroxidase conjugated (manufactured by Jackson ImmunoResearch Laboratories, Inc., #109-035-097) diluted 2500-fold with PBS containing 3% fetal bovine serum was added at 50 μl/well, and to the well to which the soluble TRAIL was added, anti-FLAG M2 monoclonal antibody-peroxidase conjugate diluted 2000-fold was added at 50 μl/well, and the plate was left to stand at room temperature for 1 hour. After PBS was added to each well, the liquid in the well was removed, and the well was washed twice with PBS. Thereafter, an OPD color developing liquid was added at 100 μl/well, whereby a color was developed. Then, 1 M HCl was added at 100 μl/well, whereby the color developing reaction was stopped. Thereafter, the absorbance at 492 nm was measured using a plate reader. Fig. 2A shows the results for cB273, and Fig. 2B shows the results for the soluble TRAIL. The data in the graphs are expressed by a mean ± standard deviation (n = 3). As a result, it was shown that cB273 selectively binds to the extracellular domain of human TRAIL R2.

3)-2 Evaluation of binding activity of cB273 antibody using Biacore

3)-2-1 Preparation of human DR5 extracellular domain protein

3)-2-1-1 Production of DR5 extracellular domain protein

expression vector

In order to construct a vector which expresses a region (hereinafter referred to as “sDR5”) composed of an amino acid sequence shown by amino acid numbers 1 to 130 of human DR5 represented by SEQ ID NO: 23 in the Sequence Listing, a PCR reaction was performed using a primer set for amplifying sDR5: DR5 Ndefw: 5′-gtggcatatggctctgatcacccaacaa-3′ (SEQ ID NO: 24 in the Sequence Listing) and
DR5 Xhorv: 5′-cgcctcgagtgattctttgtggacaca-3′ (SEQ ID NO: 25 in the Sequence Listing) and also using a cDNA encoding a human DR5 extracellular domain as a template. The resulting PCR product was cleaved with NdeI and XhoI and cloned into the NdeI/XhoI site of pET21b(+) (manufactured by Novagen, Inc.) (hereinafter abbreviated as “pET21b(+)-sDR5”). Further, a recombinant protein expressed by “pET21b(+)-sDR5” is referred to as “rsDR5” (SEQ ID NO: 26 in the Sequence Listing) hereinafter and in the drawings.

3)-2-1-2 Production of DR5 extracellular domain protein (rsDR5)

Escherichia coli Origami B (DE3) (manufactured by Novagen, Inc.) was transformed with the expression plasmid pET21b(+)-sDR5 and cultured in 2-YT medium supplemented with 100 μg/ml ampicillin (manufactured by Sigma Co., Ltd.) and 15 μg/ml kanamycin (manufactured by Wako Pure Chemical Industries,

Ltd.), and the expression of a partial protein of DR5 was induced by the addition of 0.5 mM IPTG. The cells were collected by centrifugation at 6000 rpm for 20 minutes and suspended in a binding buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl), followed by ultrasonic homogenization on ice. The resulting homogenate was centrifuged at 25000 rpm for 20 minutes. The supernatant was recovered and applied to Ni-NTA (manufactured by Invitrogen Corporation). After washing was performed with the binding buffer, elution was performed with an elution buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl and 300 mM imidazole). The eluted sample was dialyzed with a dialysis buffer (50 mM Tris-HCl pH 8.0, 20 mM NaCl) and applied to MONO Q, and gradient elution was performed with an elution buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl). The eluted sample was further purified by gel filtration column chromatography (Superdex 75 16/60, manufactured by GE Healthcare Bio-Sciences Co., Ltd.) using PBS as a solvent. The concentration of the thus obtained recombinant protein was measured at UV 280 nm (molar absorbance constant: 14855).

3)-2-2 Measurement of binding activity using Biacore

The dissociation constant of each of the cB273 antibody and rsDR5 was measured using Biacore 3000 (GE Healthcare Bio-Sciences Co., Ltd.) by a capture method in which an antibody is captured by an immobilized anti-human IgG (Fc) antibody and

the measurement is performed using an antigen as an analyte. The anti-human IgG (Fc) antibody (Human Antibody Capture Kit, GE Healthcare Bio-Sciences Co., Ltd.) was covalently immobilized onto a sensor chip CM5 (BIAcore, Inc.) at about 8000 RU by an amine coupling method. Immobilization was performed also onto a reference cell in the same manner. As a running buffer, HBS-EP (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used. Onto the chip having the anti-human IgG (Fc) antibody immobilized thereon, a 50 nM cB273 antibody solution was added at a flow rate of 10 μl/min for 60 seconds, and then, a dilution series of rsDR5 (0.63-20 nM) was added at a flow rate of 30 μl/min for 60 seconds, and subsequently, the dissociation phase was monitored for 300 seconds. As a regeneration solution, 3 M magnesium chloride was added at a flow rate of 10 μl/min for 30 seconds. In the data analysis, analysis software (BIAevaluation software, version 3.1) was used with a one-to-one binding model, and an association rate constant (kon), a dissociation rate constant (koff), and a dissociation constant (KD; KD = koff/kon) were calculated. The results obtained by the measurement using Biacore for the cB273 antibody are shown in Fig. 3.

3)-3 In vitro cytocidal effect of cB273 antibody on human cancer cell lines

The cytocidal effect of the cB273 antibody on various

cancer cell lines was studied by the following method. A2780, SK-OV-3, SK-CO-1, Caov-3, and NIH:OVCAR-5 (all of which are human ovarian cancer cell lines), HCT-15, COLO 205, HT29, SW480, SW620, DLD-1, COLO 201, and WiDr (all of which are human colon cancer cell lines), NCI-H1975, NCI-H292, NCI-H460, and NCI-H358 (all of which are human lung cancer cell lines), MDA-MB-231 and ZR-75-1 (both of which are human breast cancer cell lines) were purchased from American Type Culture Collection (ATCC). Each of these cell lines was prepared at 1×10^5 cells/ml with a medium containing 10% fetal bovine serum (manufactured by HyClone Laboratories, Inc.) (hereinafter referred to as "the medium") and seeded at 50 μl/well in a white clear bottom 96-well microplate (manufactured by Corning Incorporated). The cB273 antibody was prepared at 20000 ng/ml with a 1 μg/ml solution of a secondary antibody (goat anti-human IgG Fc, manufactured by MP Biomedicals, LLC.), and then prepared at 2000, 200, 20, and 2 ng/ml with the medium, and each of the resulting solutions was added to the plate at 50 μl/well (the final concentration of the antibody: 10000, 1000, 100, 10, and 1 ng/ml). After the plate was incubated under the conditions of 37°C and 5% CO_2 for 72 hours, the amount of ATP derived from viable cells in each well was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit (manufactured by Promega Corporation, #G7571) by a luminometer (manufactured by Berthold Technologies) according to the

attached protocol. A well to which the medium and the cell suspension were added was prepared as a negative control well and a well to which only the medium was added was prepared as a background well, and cell viability in each test well was calculated. In Fig. 4, a mean ± standard error (n = 3) of the cell viability for each cancer cell line treated with the cB273 antibody is shown. The cB273 antibody exhibited a cytocidal activity against the cell lines except for SK-CO-1.

An in vitro cytocidal effect on various cancer cell lines was studied using BxPC-3 and MIA PaCa-2 (both of which are human pancreatic cancer cell lines), A2058 and A375 (both of which are human melanoma cell lines), U-87MG (a human glioblastoma cell line), AN3CA (a human endometrial cancer cell line) as test subjects. Each of these cell lines was prepared at 4.4×10^4 cells/ml with a medium containing 10% FBS and added at 45 μl/well in a white clear bottom 96-well microplate (manufactured by Corning Incorporated), and the plate was incubated overnight under the conditions of 37°C and 5% CO_2. The cB273 antibody was mixed with the same concentration of goat anti-human IgG Fc (manufactured by MP Biomedicals, LLC.), and then the resulting mixture was added to the plate at 5 μl/well such that the final concentration of the cB273 antibody was from 10,000 to 1 ng/ml, and the plate was incubated under the conditions of 37°C and 5% CO_2 for 24 hours. The amount of ATP derived from viable cells in each well was measured using

a CellTiter-Glo Luminescent Cell Viability Assay kit (manufactured by Promega Corporation, #G7571) by a luminometer (manufactured by Perkin Elmer Inc.) according to the attached protocol. Each graph shows the cell viability expressed by a mean ± standard deviation (n = 3). As a result, the cB273 antibody exhibited a cytocidal effect on all of the cancer cell lines studied (Fig. 5).

[Example 4] Identification of epitope of cB273 antibody

4)-1 Production of cB273 Fab fragment

The cB273 was dialyzed with PBS, and then diluted to 2 mg/ml with PBS and prepared to a final volume of 17 ml. Cysteine (manufactured by Sigma Co., Ltd.) prepared at 0.1 mM with PBS in an amount of 1.7 ml and papain (manufactured by Sigma Co., Ltd.) diluted to 0.1 mg/ml with PBS in an amount of 2.04 ml were added thereto, and the reaction was allowed to proceed at 37°C for 5 hours. After 5 hours, N-ethylmaleimide (manufactured by Tokyo Chemical Industry Co., Ltd.) dissolved at 120 mM in PBS in an amount of 6.33 ml was added thereto to stop the reaction. The reaction solution was added to Superdex 200 26/60 (manufactured by GE Healthcare, Co., Ltd.) equilibrated with 50 mM Tris-HCl containing 20 mM NaCl, and 14 ml of a fraction corresponding to the Fab fragment was collected.

4)-2 Preparation of cB273 Fab fragment-rsDR5 complex sample

The cB273 Fab fragment was concentrated to 9.46 mg/ml using Amicon Ultra-15 (MWCO: 10 K) (manufactured by Millipore Co., Ltd.), and 2 ml of the thus concentrated cB273 Fab fragment was mixed with 2 ml of rsDR5 concentrated to 5.6 mg/ml using Amicon Ultra-15 (MWCO: 3 K), and the resulting mixture was added to Superdex 200 16/60 equilibrated with 20 mM Tris-HCl containing 50 mM NaCl.

8 ml of a fraction corresponding to the complex was collected.

4)-3 Crystallization and structural analysis of cB273 Fab fragment-rsDR5 complex

The thus obtained rsDR5-cB273 Fab complex was concentrated to 25 mg/ml, which was used for crystallization. For crystallization, a vapor diffusion method was used. A solution obtained by adding an equal amount of a precipitant solution (6 to 8% (w/v) polyethylene glycol 4000, 20% (v/v) isopropanol, 0.1 M lithium chloride, 0.1 M citrate buffer (pH 5.6)) to 0.45 to 1.0 μl of a protein solution was placed in an airtight container, in which 0.45 ml of the precipitant solution had already been placed, such that both solutions did not come into contact with each other, and the container was left to stand at 22°C. After 3 days, a plate-shaped crystal (0.4 mm x 0.3 mm x 0.03 mm) was obtained.

The thus obtained crystal was immersed in the precipitant solution supplemented with 30% (v/v) glycerol, and then frozen in a nitrogen stream at −180°C. X-ray diffraction data were collected in a nitrogen stream at 95 K on BL17A at the Photon Factory of the Institute of Materials Structure Science in the High Energy Accelerator Research Organization. From the obtained diffraction image, a diffraction intensity was quantified using HKL-2000 software (produced by HKL Research, Inc.), and crystal structure factors were calculated. The obtained crystal belonged to the monoclinic system, the space group was C2, and the crystal had unit-cell parameters: a = 152.0 Å, b = 75.5 Å, c = 116.3 Å, and β = 110.2.

A molecular replacement method was carried out using the thus obtained structure factors and the three-dimensional structure coordinates of DR5 (PDB code: 2H9G) and Herceptin Fab (PDB code: 1N8Z), and phases were determined. In the calculation, a software phaser (CCP4: Collaborative Computational Project No. 4) was used. The crystal contained two complexes in the asymmetric unit.

The structure was refined using CNX software (Accerlys Inc.), and the model was corrected using the Coot software. This procedure was repeated, and a final R value of 25.0% and a free R value of 28.7% were obtained at a resolution of 2.1 Å. The final model was composed of two complexes, and contains the amino acid residues 1 to 218 of the cB273 Fab L chain (both

molecules), the amino acid residues 1 to 222 of the H chain (both molecules), the amino acid residues 18 to 92 and 98 to 127 of one molecule of the DR5 among the two molecules, the amino acid residues 21 to 92 and 98 to 127 of the other molecule of the DR5, and 324 water molecules. No clear electron density was observed for the amino acid residues 93 to 97 of the DR5, 17 or 20 residues in the N terminus of the DR5, 6 residues and a His tag in the C terminus of the DR5, one residue in the C terminus of the cB273 Fab L chain and 5 residues in the C terminus of the cB273 Fab H chain, and therefore, a model was not constructed. The model was validated by a Ramachandran plot, and it was found that only Val 56 of the L chain was located outside the allowed region, and this amino acid Val 56 has a structure characteristic of CDR L2.

The interactions determined between the DR5 and cB273 Fab were substantially equal in the two molecules in the asymmetric unit. The amino acid residues (the position of each amino acid residue corresponds to that of SEQ ID NO: 23) of the DR5 which lay at a distance of 4 Å or less from the cB273 Fab were as follows: Gly26, Ile34, Glu36, Asp37, Gly38, Asp56, Leu57, Leu58, Phe59, Leu61, and Arg62. Fig. 6 shows a ribbon model of the entire complex and the surface thereof, and Fig. 7 shows the interaction between the DR5 and the H or L chain of the cB273 Fab.

[Example 5] Humanization of cB273 antibody (1)

5)-1 Designing of humanized B273 (hB273)

5)-1-1 Molecular Modeling of B273 variable regions

The molecular modeling of the B273 variable regions was performed according to a method generally known as homology modeling (Methods in Enzymology, 203, 121-153, (1991)). The primary sequences (three-dimensional structures derived from the X-ray crystal structures are available) of the variable regions of human immunoglobulin registered in Protein Data Bank (Nuc. Acid Res. 28, 235-242 (2000)) were compared with the B273 variable regions determined above. As a result, 1T66 was selected as a sequence having the highest sequence homology with the B273 light chain variable region. Further, 1XIW was selected as a sequence having the highest sequence homology with the B273 heavy chain variable region. The three-dimensional structure of a framework region was prepared based on a "framework model" by combining the coordinates of 1T66 and 1XIW corresponding to the B273 light chain and heavy chain. For the B273 CDRs, CDRL1, CDRL2, CDRL3, CDRH1, and CDRH2 were assigned to clusters 16A, 7A, 9A, 10A, and 10A, respectively, according to the classification of Thornton et al. (J. Mol. Biol., 263, 800-815, (1996)). The CDRH3 was classified in k(11)- according to the H3 rules (FEBS letters 399, 1-8 (1996)). Subsequently, the representative conformation of each CDR was incorporated in the framework

model.

Finally, in order to obtain a possible molecular model of the B273 variable region in terms of energy, an energy calculation was performed for excluding disadvantageous interatomic contact. The above procedure was carried out using the commercially available protein tertiary structure prediction program Prime and coordinate search program MacroModel (Schrödinger, LLC).

5)-1-2 Designing of amino acid sequence of humanized B273

A humanized B273 antibody was constructed according to a method generally known as CDR grafting (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)). An acceptor antibody was selected based on the amino acid homology within the framework region.

The sequence of the framework region of B273 was compared with all human framework sequences in the Kabat Database (Nuc. Acid Res. 29, 205-206 (2001)) of antibody amino acid sequences. As a result, a HuMc3 antibody was selected as an acceptor based on a sequence homology of 76% in the framework region. The amino acid residues in the framework region of HuMc3 were aligned with the amino acid residues of B273, and the positions where different amino acids were used were identified. The positions of these residues were analyzed using the three-dimensional model of B273 constructed above. Then,

donor residues to be grafted onto the acceptor were selected according to the criteria provided by Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)).

By transferring some selected donor residues to the acceptor antibody, humanized B273 sequences were constructed as described in the following Example.

5)-1-2 Humanization of B273 light chain

5)-1-2-1 hB273_L1-type light chain:

A humanized B273 light chain designed by substituting amino acid numbers 22 (valine), 27 (threonine), 34 (serine), 35 (leucine), 37 (aspartic acid), 38 (glutamine), 70 (lysine), 108 (leucine), 110 (isoleucine), 112 (phenylalanine), 125 (glycine), and 129 (leucine) of the cB273 light chain represented by SEQ ID NO: 16 in the Sequence Listing with isoleucine, serine, threonine, proline, glutamic acid, proline, glutamine, valine, valine, tyrosine, proline, and valine, respectively, was named “hB273_L1-type light chain”.

A nucleotide sequence encoding the hB273_L1-type light chain is represented by SEQ ID NO: 27 in the Sequence Listing. Further, the amino acid sequence of the hB273_L1-type light chain is represented by SEQ ID NO: 28 in the Sequence Listing. The sequences of SEQ ID NOS: 27 and 28 are also shown in Fig. 32.

5)-1-2-2 hB273_L2-type light chain:

A humanized B273 light chain designed by substituting amino acid numbers 37 (aspartic acid), 38 (glutamine), 108 (leucine), 110 (isoleucine), 125 (glycine), and 129 (leucine) of the cB273 light chain represented by SEQ ID NO: 16 in the Sequence Listing with glutamic acid, proline, valine, valine, proline, and valine, respectively, was named “hB273_L2-type light chain”.

A nucleotide sequence encoding the hB273_L2-type light chain is represented by SEQ ID NO: 29 in the Sequence Listing. Further, the amino acid sequence of the hB273_L2-type light chain is represented by SEQ ID NO: 30 in the Sequence Listing. The sequences of SEQ ID NOS: 29 and 30 are also shown in Fig. 33.

5)-1-2-3 hB273_L3-type light chain:

A humanized B273 light chain designed by substituting amino acid numbers 37 (aspartic acid), 38 (glutamine), 108 (leucine), 110 (isoleucine), and 129 (leucine) of the cB273 light chain represented by SEQ ID NO: 16 in the Sequence Listing with glutamic acid, proline, valine, valine, and valine, respectively, was named “hB273_L3-type light chain”.

A nucleotide sequence encoding the hB273_L3-type light chain is represented by SEQ ID NO: 31 in the Sequence Listing. Further, the amino acid sequence of the hB273_L3-type light

chain is represented by SEQ ID NO: 32 in the Sequence Listing. The sequences of SEQ ID NOS: 31 and 32 are also shown in Fig. 34.

5)-1-3 Humanization of B273 heavy chain

5)-1-3-1 hB273_H1-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 56 (methionine), 57 (lysine), 59 (serine), 60 (histidine), 62 (lysine), 63 (serine), 67 (isoleucine), 86 (lysine), 87 (alanine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 114 (phenylalanine), 116 (glycine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, valine, arginine, alanine, proline, methionine, glycine, methionine, arginine, valine, threonine, serine, tyrosine, serine, arginine, threonine, tyrosine, alanine, leucine, and valine, respectively, was named “hB273_H1-type heavy chain”.

A nucleotide sequence encoding the hB273_H1-type heavy chain is represented by SEQ ID NO: 33 in the Sequence Listing. Further, the amino acid sequence of the hB273_H1-type heavy chain is represented by SEQ ID NO: 34 in the Sequence Listing. The sequences of SEQ ID NOS: 33 and 34 are also shown in Fig.

35.

5)-1-3-2 hB273_H2-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 62 (lysine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 116 (glycine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, proline, methionine, threonine, serine, tyrosine, serine, arginine, threonine, alanine, leucine, and valine, respectively, was named “hB273_H2-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-type heavy chain is represented by SEQ ID NO: 35 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-type heavy chain is represented by SEQ ID NO: 36 in the Sequence Listing. The sequences of SEQ ID NOS: 35 and 36 are also shown in Fig. 36.

5)-1-3-3 hB273_H3-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 95 (serine), 96

(threonine), 103 (leucine), 106 (threonine), 110 (serine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with valine, alanine, valine, lysine, valine, proline, threonine, serine, serine, arginine, threonine, leucine, and valine, respectively, was named “hB273_H3-type heavy chain”.

A nucleotide sequence encoding the hB273_H3-type heavy chain is represented by SEQ ID NO: 37 in the Sequence Listing. Further, the amino acid sequence of the hB273_H3-type heavy chain is represented by SEQ ID NO: 38 in the Sequence Listing. The sequences of SEQ ID NOS: 37 and 38 are also shown in Fig. 37.

5)-2 Construction of hB273_L1, hB273_L2, and hB273_L3-type light chain expression vectors

DNAs containing a gene encoding a hB273_L1, hB273_L2, or hB273_L3-type light chain variable region represented by amino acid numbers 21 to 134 of SEQ ID NO: 28, amino acid numbers 21 to 134 of SEQ ID NO: 30, or amino acid numbers 21 to 134 of SEQ ID NO: 32 were synthesized (GENEART, Inc. Artificial Gene Synthesis Service). Then, each of the DNA fragments obtained by cleaving the synthesized DNAs with the restriction enzymes NdeI and BsiWI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NdeI and BsiWI,

whereby hB273_L1, hB273_L2, and hB273_L3-type light chain expression vectors were constructed. The thus obtained expression vectors were named “pEF6KCL/hB273_L1”, “pEF6KCL/hB273_L2”, and “pEF6KCL/hB273_L3”, respectively.

5)-3 Construction of hB273_H1, hB273_H2, and hB273_H3-type heavy chain expression vectors

DNAs containing a gene encoding a hB273_H1, hB273_H2, or hB273_H3-type heavy chain variable region represented by amino acid numbers 20 to 141 of SEQ ID NO: 34 in the Sequence Listing, amino acid numbers 20 to 141 of SEQ ID NO: 36, or amino acid numbers 20 to 141 of SEQ ID NO: 38 were synthesized (GENEART, Inc. Artificial Gene Synthesis Service). Then, each of the DNA fragments obtained by cleaving the synthesized DNAs with the restriction enzyme BlpI was inserted into the universal humanized antibody heavy chain expression vector (pEF1FCCU) at the site cleaved with the restriction enzyme BlpI, whereby hB273_H1, hB273_H2, and hB273_H3-type heavy chain expression vectors were constructed. The thus obtained expression vectors were named “pEF1FCCU/hB273_H1”, “pEF1FCCU/hB273_H2”, and “pEF1FCCU/hB273_H3”, respectively.

5)-4 Preparation of humanized antibody

5)-4-1 Production of humanized antibody

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen

Corporation) in the logarithmic growth phase were seeded into 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen Corporation) and cultured for 1 hour by shaking at 90 rpm at 37°C in an 8% CO_2 incubator. 3.6 mg of polyethyleneimine (Polyscience #24765) was dissolved in 20 ml of Opti-Pro SFM medium (Invitrogen Corporation). Subsequently, a heavy chain expression vector (0.4 mg) and a light chain expression vector (0.8 mg) prepared with PureLink HiPure Plasmid Kit (Invitrogen Corporation) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the obtained expression vectors/Opti-Pro SFM mixed liquid was added to 20 ml of the obtained polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells. After 3 hours, Z-VAD-FMK (PEPTIDE INSTITUTE, Inc.) was added thereto at a final concentration of 10 μM, and culture shaking at 90 rpm was performed for 7 days at 37°C in an 8% CO_2 incubator. The resulting culture supernatant was filtered through a disposable capsule filter (Advantec #CCS-045-E1H).

A humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H1 and pEF6KCL/hB273_L1 was named "hB273_H1/hB273_L1", a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H1 and pEF6KCL/hB273_L2 was named "hB273_H1/hB273_L2", a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H1 and

pEF6KCL/hB273_L3 was named “hB273_H1/hB273_L3”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2 and pEF6KCL/hB273_L1 was named “hB273_H2/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2 and pEF6KCL/hB273_L2 was named “hB273_H2/hB273_L2”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2 and pEF6KCL/hB273_L3 was named “hB273_H2/hB273_L3”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H3 and pEF6KCL/hB273_L1 was named “hB273_H3/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H3 and pEF6KCL/hB273_L2 was named “hB273_H3/hB273_L2”, and a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H3 and pEF6KCL/hB273_L3 was named “hB273_H3/hB273_L3”.

5)-4-2 Purification of humanized antibody

The culture supernatant obtained in the above 5)-4-1 was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE

Healthcare Bio-Sciences Co., Ltd., two HiTrap columns (volume: 1 ml) connected in series) equilibrated with PBS. After all culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0), and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the buffer was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to buffer replacement was applied to a ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1 hydroxyapatite column (volume: 2 ml)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), and the

concentration of IgG was adjusted to 1.0 mg/ml or more, and the thus obtained solution was used as a purified sample.

[Example 6] Measurement of activity of humanized B273 (hB273) antibody (1)

6)-1 Evaluation of binding activity of hB273 antibody using Biacore

The dissociation constant of each of the humanized anti-DR5 antibodies and rsDR5 was measured using Biacore T100 (GE Healthcare Bio-Sciences Co., Ltd.) by a capture method in which an antibody is captured by an immobilized anti-human IgG (Fc) antibody and the measurement is performed using an antigen as an analyte. The anti-human IgG (Fc) antibody (Human Antibody Capture Kit, GE Healthcare Bio-Sciences Co., Ltd.) was covalently immobilized onto a sensor chip CM5 (BIAcore, Inc.) at about 10,000 RU by an amine coupling method. Immobilization was also performed onto a reference cell in the same manner. As a running buffer, HBS-EP (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used. Onto the chip having the anti-human IgG (Fc) antibody immobilized thereon, an antibody solution at about 20 nM was added at a flow rate of 10 μl/min for 60 seconds, and then, a dilution series of rsDR5 (3.13-50 nM) was added at a flow rate of 30 μl/min for 120 seconds, and subsequently, the dissociation phase was monitored for 180 seconds. As a

regeneration solution, 3 M magnesium chloride was added at a flow rate of 10 μl/min for 30 seconds. In the data analysis, analysis software (Biacore T100 Evaluation software, version 2.0.1) was used with a one-to-one binding model, and an association rate constant (kon), a dissociation rate constant (koff), and a dissociation constant (KD; KD = koff/kon) were calculated. The results obtained by the measurement using Biacore for the 9 types of humanized DR5 antibodies are shown in Fig. 8.

6)-2 In vitro cytocidal activity of hB273 antibody against human cancer cell line

AffiniPure $F(ab')_2$ fragment goat anti-human IgG Fc fragment specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #109-006-098) prepared at 50 μg/ml with 50 mM Tris-HCl (pH 8.5) was dispensed at 45 μL/well in a 96-well microplate (manufactured by Corning Incorporated), and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, the culture supernatant of 293F which was allowed to produce the antibody in 5)-4-1, the purified cB273 antibody (Example 2-3-2), or commercially available human IgG (manufactured by Jackson ImmunoResearch Laboratories, Inc., #009-000-003) was added at 50 μL/well such that the final concentration of the antibody was from 150 to 1.5 ng/ml, and the plate was left to stand overnight at 4°C.

After each well was washed twice with PBS, Jurkat cells prepared at 4.0 x 10^4 cells/ml in RPMI 1640 medium containing 10% FBS were added at 50 μl/well and cultured under the conditions of 37°C and 5% CO_2 for 23 hours. The amount of ATP derived from viable cells was quantitatively determined using a CellTiter-Glo luminescent cell viability assay kit (manufactured by Promega Corporation, #G7571), and the cytocidal effect of each of the hB273 antibodies was evaluated by taking the value obtained from a well to which the medium was added in place of the antibody solution as 100%. As a result, as shown in Fig. 9, with respect to the humanization of the B273 heavy chain, a tendency was observed that the antibodies comprising the H1-type heavy chain exhibited a slightly lower cytocidal effect than the antibodies comprising the H2 or H3-type heavy chain. On the other hand, with respect to the humanization of the B273 light chain, it was found that the antibodies comprising any of the designed L1, L2, and L3-type light chains can exhibit substantially the same cytocidal effect.

[Example 7] Humanization of the cB273 antibody (2)

7)-1 Designing of humanized B273 (hB273)

7)-1-1 Designing of amino acid sequence of humanized B273

Based on the results of the designing of the humanized antibodies (1) shown in Examples 5 and 6, by transferring some

donor residues to the acceptor antibody, humanized B273 sequences were constructed as described in the following Example.

7)-1-2 Designing of amino acid sequence of humanized B273

7)-1-2-1 hB273_H1-1-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 57 (lysine), 59 (serine), 60 (histidine), 62 (lysine), 63 (serine), 67 (isoleucine), 86 (lysine), 87 (alanine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, arginine, alanine, proline, methionine, glycine, methionine, arginine, valine, threonine, serine, tyrosine, serine, arginine, threonine, leucine, and valine, respectively, was named “hB273_H1-1-type heavy chain”.

A nucleotide sequence encoding the hB273_H1-1-type heavy chain is represented by SEQ ID NO: 39 in the Sequence Listing. Further, the amino acid sequence of the hB273_H1-1-type heavy chain is represented by SEQ ID NO: 40 in the Sequence Listing. The sequences of SEQ ID NOS: 39 and 40 are also shown in Fig. 38.

7)-1-2-2 hB273_H2-1-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 62 (lysine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 116 (glycine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with valine, alanine, valine, lysine, valine, proline, methionine, threonine, serine, tyrosine, serine, arginine, threonine, alanine, leucine, and valine, respectively, was named “hB273_H2-1-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-1-type heavy chain is represented by SEQ ID NO: 41 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-1-type heavy chain is represented by SEQ ID NO: 42 in the Sequence Listing. The sequences of SEQ ID NOS: 41 and 42 are also shown in Fig. 39.

7)-1-2-3 hB273_H2-2-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 62 (lysine), 95 (serine), 96 (threonine), 103

(leucine), 106 (threonine), 110 (serine), 116 (glycine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, proline, methionine, threonine, serine, serine, arginine, threonine, alanine, leucine, and valine, respectively, was named “hB273_H2-2-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-2-type heavy chain is represented by SEQ ID NO: 43 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-2-type heavy chain is represented by SEQ ID NO: 44 in the Sequence Listing. The sequences of SEQ ID NOS: 43 and 44 are also shown in Fig. 40.

7)-1-2-4 hB273_H2-3-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 62 (lysine), 95 (serine), 96 (threonine), 103 (leucine), 106 (threonine), 110 (serine), 116 (glycine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with valine, alanine, valine, lysine, valine, proline, methionine, threonine, serine, serine, arginine, threonine, alanine, leucine, and valine, respectively, was named “hB273_H2-3-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-3-type heavy chain is represented by SEQ ID NO: 45 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-3-type heavy chain is represented by SEQ ID NO: 46 in the Sequence Listing. The sequences of SEQ ID NOS: 45 and 46 are also shown in Fig. 41.

7)-1-2-5 hB273_H2-4-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 62 (lysine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, proline, methionine, threonine, serine, tyrosine, serine, arginine, threonine, leucine, and valine, respectively, was named “hB273_H2-4-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-4-type heavy chain is represented by SEQ ID NO: 47 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-4-type heavy chain is represented by SEQ ID NO: 48 in the Sequence Listing. The sequences of SEQ ID NOS: 47 and 48 are also shown in Fig. 42.

7)-1-2-6 hB273_H2-5-type heavy chain:

A humanized B273 heavy chain designed by substituting amino acid numbers 20 (glutamic acid), 24 (glutamine), 28 (proline), 30 (leucine), 31 (valine), 39 (isoleucine), 60 (histidine), 95 (serine), 96 (threonine), 99 (histidine), 103 (leucine), 106 (threonine), 110 (serine), 136 (threonine), and 137 (leucine) of the cB273 heavy chain represented by SEQ ID NO: 20 in the Sequence Listing with glutamine, valine, alanine, valine, lysine, valine, proline, threonine, serine, tyrosine, serine, arginine, threonine, leucine, and valine, respectively, was named “hB273_H2-5-type heavy chain”.

A nucleotide sequence encoding the hB273_H2-5-type heavy chain is represented by SEQ ID NO: 49 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-5-type heavy chain is represented by SEQ ID NO: 50 in the Sequence Listing. The sequences of SEQ ID NOS: 49 and 50 are also shown in Fig. 43.

7)-2 Construction of hB273_H1-1, hB273_H2-1, hB273_H2-2, hB273_H2-3, hB273_H2-4, and hB273_H2-5-type heavy chain expression vectors

DNAs containing a gene encoding a hB273_H1-1, hB273_H2-1, hB273_H2-2, hB273_H2-3, hB273_H2-4, or hB273_H2-5-type heavy chain variable region represented by amino acid numbers 20 to 141 of SEQ ID NO: 40 in the Sequence

Listing, amino acid numbers 20 to 141 of SEQ ID NO: 42, amino acid numbers 20 to 141 of SEQ ID NO: 44, amino acid numbers 20 to 141 of SEQ ID NO: 46, amino acid numbers 20 to 141 of SEQ ID NO: 48, or amino acid numbers 20 to 141 of SEQ ID NO: 50 were synthesized (GENEART, Inc. Artificial Gene Synthesis Service). Then, each of the DNA fragments obtained by cleaving the synthesized DNAs with the restriction enzyme BlpI was inserted into the universal humanized antibody heavy chain expression vector (pEF1FCCU) at the site cleaved with the restriction enzyme BlpI, whereby hB273_H1-1, hB273_H2-1, hB273_H2-2, hB273_H2-3, hB273_H2-4, and hB273_H2-5-type heavy chain expression vectors were constructed. The thus obtained expression vectors were named “pEF1FCCU/hB273_H1-1”, “pEF1FCCU/hB273_H2-1”, “pEF1FCCU/hB273_H2-2”, “pEF1FCCU/hB273_H2-3”, “pEF1FCCU/hB273_H2-4”, and “pEF1FCCU/hB273_H2-5”, respectively.

7)-3 Preparation of humanized antibody

7)-3-1 Production of humanized antibody

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen Corporation) in the logarithmic growth phase were seeded into 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen Corporation) and cultured for 1 hour by shaking at 90 rpm at 37°C in an 8% CO_2 incubator. 3.6 mg of polyethyleneimine (Polyscience #24765) was dissolved in 20 ml of Opti-Pro SFM

medium (Invitrogen Corporation). Subsequently, a heavy chain expression vector (0.4 mg) and a light chain expression vector (0.8 mg) prepared with PureLink HiPure Plasmid Kit (Invitrogen Corporation) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the obtained expression vectors/Opti-Pro SFM mixed liquid was added to 20 ml of the obtained polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells. After 3 hours, Z-VAD-FMK (PEPTIDE INSTITUTE, Inc.) was added thereto at a final concentration of 10 μM, and culture shaking at 90 rpm was performed for 7 days at 37°C in an 8% CO_2 incubator. The resulting culture supernatant was filtered through a disposable capsule filter (Advantec #CCS-045-E1H).

A humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H1-1 and pEF6KCL/hB273_L1 was named “hB273_H1-1/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-1 and pEF6KCL/hB273_L1 was named “hB273_H2-1/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-2 and pEF6KCL/hB273_L1 was named “hB273_H2-2/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-3 and pEF6KCL/hB273_L1 was named “hB273_H2-3/hB273_L1”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-4 and pEF6KCL/hB273_L1

was named “hB273_H2-4/hB273_L1”, and a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-5 and pEF6KCL/hB273_L1 was named “hB273_H2-5/hB273_L1”.

7)-3-2 Purification of humanized antibody

The culture supernatant obtained in the above 7)-3-1 was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap columns (volume: 1 ml) connected in series) equilibrated with PBS. After all the culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0), and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the buffer was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to buffer replacement was applied to a

ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1 hydroxyapatite column (volume: 2 ml)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), and the concentration of IgG was adjusted to 1.0 mg/ml or more, and the thus obtained solution was used as a purified sample.

[Example 8] Measurement of activity of humanized B273 (hB273) antibody (2)

8)-1 Evaluation of binding activity of hB273 antibody using Biacore

The dissociation constant of each of the humanized anti-DR5 antibodies and rsDR5 was measured using Biacore T100 (GE Healthcare Bio-Sciences Co., Ltd.) by a capture method in which an antibody is captured by an immobilized anti-human IgG (Fc) antibody and the measurement is performed using an antigen

as an analyte. The anti-human IgG (Fc) antibody (Human Antibody Capture Kit, GE Healthcare Bio-Sciences Co., Ltd.) was covalently immobilized onto a sensor chip CM5 (BIAcore, Inc.) at about 10,000 RU by an amine coupling method. Immobilization was also performed onto a reference cell in the same manner. As a running buffer, HBS-EP (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used. Onto the chip having the anti-human IgG (Fc) antibody immobilized thereon, an antibody solution at about 20 nM was added at a flow rate of 10 μl/min for 60 seconds, and then, a dilution series of rsDR5 (3.13-50 nM) was added at a flow rate of 30 μl/min for 120 seconds, and subsequently, the dissociation phase was monitored for 180 seconds. As a regeneration solution, 3 M magnesium chloride was added at a flow rate of 10 μl/min for 30 seconds. In the data analysis, analysis software (Biacore T100 Evaluation software, version 2.0.1) was used with a one-to-one binding model, and an association rate constant (kon), a dissociation rate constant (koff), and a dissociation constant (KD; KD = koff/kon) were calculated. The results obtained by the measurement using Biacore for the 6 types of humanized DR5 antibodies are shown in Fig. 10.

8)-2 In vitro cytocidal activity of hB273 antibody against human cancer cell line

AffiniPure F(ab')$_2$ fragment goat anti-human IgG Fc fragment specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #109-006-098) prepared at 50 μg/ml with 50 mM Tris-HCl (pH 8.5) was dispensed at 45 μL/well in a 96-well microplate (manufactured by Corning Incorporated), and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, the culture supernatant of 293F which was allowed to produce the antibody in 7)-3-1 was added at 50 μL/well such that the final concentration of the antibody was from 150 to 1.5 ng/ml, and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, Jurkat cells prepared at 4.0 x 10^4 cells/ml in RPMI 1640 medium containing 10% FBS were added at 50 μl/well and cultured under the conditions of 37°C and 5% CO_2 for 23 hours. The amount of ATP derived from viable cells was quantitatively determined using a CellTiter-Glo luminescent cell viability assay kit (manufactured by Promega Corporation, #G7571), and the cytocidal effect of each of the hB273 antibodies was evaluated by taking the value obtained from a well to which the medium was added in place of the antibody solution as 100%. As a result, as shown in Fig. 11, a tendency was observed for the hB273_H1-1/L1 antibody to exhibit a higher cytocidal activity than the hB273_H1/L1 antibody, on the basis of which antibody the hB273_H1-1/L1 antibody had been designed. On the other hand, the hB273_H2-1/L1 to hB273_H2-5/L1 antibodies exhibited

substantially the same cytocidal effect as the hB273_H2/L1 antibody, on the basis of which antibody the hB273_H2-1/L1 to hB273_H2-5/L1 antibodies had been designed.

[Example 9] Removal of deamidation site from cB273 antibody CDR

9)-1 Designing of mutant and construction of expression vector

9)-1-1 Designing of mutant

In general, the deamidation of asparagine in a protein proceeds through the formation of a transition state of cyclic succinimide between the asparagine and an adjacent amino acid on the C-terminal side (Geiger, T. and Clarke, S. (1987) Deamidation, Isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J. Biol. Chem. 262, 785-794). A rate-limiting factor for the formation of a transition state of cyclic succinimide is the size of the side chain of the adjacent amino acid, and therefore, glycine which has the smallest side chain can achieve the fastest deamidation rate. On the other hand, by substituting the adjacent group on the C-terminal side with an amino acid having a large side chain, the deamidation rate can be suppressed. The B273 antibody has a -N-G- (asparagine-glycine) sequence which is susceptible to deamidation in both the L chain and the H chain. Therefore, the present inventors produced point

mutants in which the adjacent group was changed from glycine to lysine, phenylalanine, leucine, or glutamic acid, each of which has a larger side chain than glycine. That is, designing of mutants was performed such that in the H chain, the -N-G- (asparagine-glycine) sequence was mutated to a -N-E- (asparagine-glutamic acid) sequence, and in the L chain, the -N-G- (asparagine-glycine) sequence was mutated to a -N-L- (asparagine-leucine) sequence, a -N-F- (asparagine-phenylalanine) sequence, a -N-K- (asparagine-lysine) sequence, or a -N-E- (asparagine-glutamic acid) sequence.

9)-1-2 Construction of hB273_L1-NE-type light chain expression vector

By using pEF6KCL/hB273_L1 which is the hB273_L1-type light chain expression vector produced in Example 5 as a template, a DNA fragment obtained by performing PCR using a primer set A and a DNA fragment obtained by performing PCR using a primer set B were ligated to each other by overlap extension PCR using a primer set C. A DNA fragment obtained by cleaving the thus obtained DNA fragment with the restriction enzymes NheI and PmeI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NheI and PmeI, whereby a hB273_L1-NE-type light chain expression vector in which

glycine at amino acid number 54 of SEQ ID NO: 28 was substituted with glutamic acid was constructed. The thus obtained expression vector was named “pEF6KCL/hB273_L1-NE”.

A nucleotide sequence encoding the hB273_L1-NE-type light chain is represented by SEQ ID NO: 51 in the Sequence Listing. Further, the amino acid sequence of the hB273_L1-NE-type light chain is represented by SEQ ID NO: 52 in the Sequence Listing. The sequences of SEQ ID NOS: 51 and 52 are also shown in Fig. 44.

Primer set A

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ccaatgcaggtaagtgttctcattgctatggaccagtgactg-3′ (L-NE-R2: SEQ ID NO: 54 in the Sequence Listing)

Primer set B

5′-cagtcactggtccatagcaatgagaacacttacctgcattgg-3′ (L-NE-F2: SEQ ID NO: 55 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

Primer set C

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

9)-1-3 Construction of hB273_L1-NF-type light chain expression vector

By using pEF6KCL/hB273_L1 which is the hB273_L1-type light chain expression vector produced in Example 5 as a template, a DNA fragment obtained by performing PCR using a primer set A and a DNA fragment obtained by performing PCR using a primer set B were ligated to each other by overlap extension PCR using a primer set C. A DNA fragment obtained by cleaving the thus obtained DNA fragment with the restriction enzymes NheI and PmeI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NheI and PmeI, whereby a hB273_L1-NF-type light chain expression vector in which glycine at amino acid number 54 of SEQ ID NO: 28 was substituted with phenylalanine was constructed. The thus obtained expression vector was named “pEF6KCL/hB273_L1-NF”.

A nucleotide sequence encoding the hB273_L1-NF-type light chain is represented by SEQ ID NO: 57 in the Sequence Listing. Further, the amino acid sequence of the hB273_L1-NF-type light chain is represented by SEQ ID NO: 58 in the Sequence Listing. The sequences of SEQ ID NOS: 57 and 58 are also shown in Fig. 45.

Primer set A

5’-aggtaagcttgctagcgccaccatggtgctgc-3’ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ccaatgcaggtaagtgttgaaattgctatggaccagtgactg-3′ (L-NF-R2: SEQ ID NO: 59 in the Sequence Listing)

Primer set B

5′-cagtcactggtccatagcaatttcaacacttacctgcattgg-3′ (L-NF-F2: SEQ ID NO: 60 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

Primer set C

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

9)-1-4 Construction of hB273_L1-NK-type light chain expression vector

By using pEF6KCL/hB273_L1 which is the hB273_L1-type light chain expression vector produced in Example 5 as a template, a DNA fragment obtained by performing PCR using a primer set A and a DNA fragment obtained by performing PCR using a primer set B were ligated to each other by overlap extension PCR using a primer set C. A DNA fragment obtained by cleaving the thus obtained DNA fragment with the restriction enzymes NheI and PmeI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NheI and PmeI, whereby

a hB273_L1-NK-type light chain expression vector in which glycine at amino acid number 54 of SEQ ID NO: 28 was substituted with lysine was constructed. The thus obtained expression vector was named “pEF6KCL/hB273_L1-NK”.

A nucleotide sequence encoding the hB273_L1-NK-type light chain is represented by SEQ ID NO: 61 in the Sequence Listing. Further, the amino acid sequence of the hB273_L1-NK-type light chain is represented by SEQ ID NO: 62 in the Sequence Listing. The sequences of SEQ ID NOS: 61 and 62 are also shown in Fig. 46.

Primer set A

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ccaatgcaggtaagtgttcttattgctatggaccagtgactg-3′ (L-NK-R2: SEQ ID NO: 63 in the Sequence Listing)

Primer set B

5′-cagtcactggtccatagcaataagaacacttacctgcattgg-3′ (L-NK-F2: SEQ ID NO: 64 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

Primer set C

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

9)-1-5 Construction of hB273_L1-NL-type light chain expression vector

By using pEF6KCL/hB273_L1 which is the hB273_L1-type light chain expression vector produced in Example 5 as a template, a DNA fragment obtained by performing PCR using a primer set A and a DNA fragment obtained by performing PCR using a primer set B were ligated to each other by overlap extension PCR using a primer set C. A DNA fragment obtained by cleaving the thus obtained DNA fragment with the restriction enzymes NheI and PmeI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NheI and PmeI, whereby a hB273_L1-NL-type light chain expression vector in which glycine at amino acid number 54 of SEQ ID NO: 28 was substituted with leucine was constructed. The thus obtained expression vector was named “pEF6KCL/hB273_L1-NL”.

A nucleotide sequence encoding the hB273_L1-NL-type light chain is represented by SEQ ID NO: 65 in the Sequence Listing. Further, the amino acid sequence of the hB273_L1-NL-type light chain is represented by SEQ ID NO: 66 in the Sequence Listing. The sequences of SEQ ID NOS: 65 and 66 are also shown in Fig. 47.

Primer set A

5’-aggtaagcttgctagcgccaccatggtgctgc-3’ (L-F1: SEQ ID NO: 53

in the Sequence Listing)

5′-ccaatgcaggtaagtgttcagattgctatggaccagtgactg-3′ (L-NL-R2: SEQ ID NO: 67 in the Sequence Listing)

Primer set B

5′-cagtcactggtccatagcaatctgaacacttacctgcattgg-3′ (L-NL-F2: SEQ ID NO: 68 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

Primer set C

5′-aggtaagcttgctagcgccaccatggtgctgc-3′ (L-F1: SEQ ID NO: 53 in the Sequence Listing)

5′-ggatgccacccgtttaaacgggcccctaacac-3′ (L-R1: SEQ ID NO: 56 in the Sequence Listing)

9)-1-6 Construction of hB273_H2-1-NE-type heavy chain expression vector

By using pEF1FCCU/hB273_H2-1 which is the hB273_H2-1-type heavy chain expression vector produced in Example 7 as a template, a DNA fragment obtained by performing PCR using a primer set A and a DNA fragment obtained by performing PCR using a primer set B were ligated to each other by overlap extension PCR using a primer set C. A DNA fragment obtained by cleaving the thus obtained DNA fragment with the restriction enzymes NheI and PmeI was inserted into the universal humanized antibody heavy chain expression vector

(pEF1FCCU) at the site cleaved with the restriction enzymes NheI and PmeI, whereby a hB273_H2-1-NE-type heavy chain expression vector in which glycine at amino acid number 75 of SEQ ID NO: 42 was substituted with glutamic acid was constructed. The thus obtained expression vector was named "pEF1FCCU/hB273_H2-1-NE".

A nucleotide sequence encoding the hB273_H2-1-NE-type heavy chain is represented by SEQ ID NO: 69 in the Sequence Listing. Further, the amino acid sequence of the hB273_H2-1-NE-type heavy chain is represented by SEQ ID NO: 70 in the Sequence Listing. The sequences of SEQ ID NOS: 69 and 70 are also shown in Fig. 48.

Primer set A

5'-aggtaagcttgctagcgccaccatgaaacacc-3' (H-F1: SEQ ID NO: 71 in the Sequence Listing)

5'-ctggttgtagaaggtgtcctcgttgtaggggttgaaccggcc-3' (H-NE-R2: SEQ ID NO: 72 in the Sequence Listing)

Primer set B

5'-ggccggttcaacccctacaacgaggacaccttctacaaccag-3' (H-NE-F2: SEQ ID NO: 73 in the Sequence Listing)

5'-ggatgccacccgtttaaacgggcccgatatctc-3' (H-R1: SEQ ID NO: 74 in the Sequence Listing)

Primer set C

5'-aggtaagcttgctagcgccaccatgaaacacc-3' (H-F1: SEQ ID NO: 71 in the Sequence Listing)

5'-ggatgccacccgtttaaacgggcccgatatctc-3' (H-R1: SEQ ID NO: 74 in the Sequence Listing)

9)-2 Preparation of CDR-modified hB273 antibody

9)-2-1 Production of CDR-modified hB273 antibody

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen Corporation) in the logarithmic growth phase were seeded into 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen Corporation) and cultured for 1 hour by shaking at 90 rpm at 37°C in an 8% CO_2 incubator. 3.6 mg of polyethyleneimine (Polyscience #24765) was dissolved in 20 ml of Opti-Pro SFM medium (Invitrogen Corporation). Subsequently, a heavy chain expression vector (0.4 mg) and a light chain expression vector (0.8 mg) prepared with PureLink HiPure Plasmid Kit (Invitrogen Corporation) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the obtained expression vectors/Opti-Pro SFM mixed liquid was added to 20 ml of the obtained polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells. After 3 hours, Z-VAD-FMK (PEPTIDE INSTITUTE, Inc.) was added thereto at a final concentration of 10 μM, and culture shaking at 90 rpm was performed for 7 days at 37°C in an 8% CO_2 incubator. The resulting culture supernatant was filtered through a disposable capsule filter (Advantec #CCS-045-E1H).

A humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-1-NE and pEF6KCL/hB273_L1-NE was named “hB273_H2-1-NE/hB273_L1-NE”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-1-NE and pEF6KCL/hB273_L1-NF was named “hB273_H2-1-NE/hB273_L1-NF”, a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-1-NE and pEF6KCL/hB273_L1-NK was named “hB273_H2-1-NE/hB273_L1-NK”, and a humanized antibody of cB273 obtained by a combination of pEF1FCCU/hB273_H2-1-NE and ppEF6KCL/hB273_L1-NL was named “hB273_H2-1-NE/hB273_L1-NL”.

9)-2-2 Purification of CDR-modified hB273 antibody

The culture supernatant obtained in the above 9)-2-1 was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap columns (volume: 1 ml) connected in series) equilibrated with PBS. After all the culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0),

and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the buffer was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to buffer replacement was applied to a ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1 hydroxyapatite column (volume: 2 ml)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), and the concentration of IgG was adjusted to 1.0 mg/ml or more, and the thus obtained solution was used as a purified sample.

[Example 10] Measurement of activity of CDR-modified hB273 antibody

10)-1 Evaluation of binding activity of CDR-modified hB273 antibody using Biacore

The dissociation constant of each of the humanized anti-DR5 antibodies and rsDR5 was measured using Biacore T100 (GE Healthcare Bio-Sciences Co., Ltd.) by a capture method in which an antibody is captured by an immobilized anti-human IgG (Fc) antibody and the measurement is performed using an antigen as an analyte. The anti-human IgG (Fc) antibody (Human Antibody Capture Kit, GE Healthcare Bio-Sciences Co., Ltd.) was covalently immobilized onto a sensor chip CM5 (BIAcore, Inc.) at about 10,000 RU by an amine coupling method. Immobilization was performed also onto a reference cell in the same manner. As a running buffer, HBS-EP (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used. Onto the chip having the anti-human IgG (Fc) antibody immobilized thereon, an antibody solution at about 20 nM was added at a flow rate of 10 μl/min for 60 seconds, and then, a dilution series of rsDR5 (3.13-50 nM) was added at a flow rate of 30 μl/min for 120 seconds, and subsequently, the dissociation phase was monitored for 180 seconds. As a regeneration solution, 3 M magnesium chloride was added at a flow rate of 10 μl/min for 30 seconds. In the data analysis, analysis software (Biacore T100 Evaluation software, version 2.0.1) was used with a one-to-one binding model, and an association rate constant (kon), a dissociation rate constant

(koff), and a dissociation constant (KD; KD = koff/kon) were calculated. The results obtained by the measurement using Biacore for the 4 types of humanized DR5 antibodies are shown in Fig. 12.

10)-2 Measurement of thermal stability of humanized anti-DR5 antibody and mutant thereof using differential scanning calorimetry (DSC)

The measurement of thermal stability was performed using differential scanning calorimetry (DSC). A sample was dissolved in a CBS buffer (containing 10 mM citric acid and 140 mM NaCl and prepared at pH 6.0) at 0.5 mg/ml, and 400 μl aliquots thereof were used as sample solutions for DSC measurement. The DSC measurement conditions were set as follows: initial temperature: 20°C; final temperature: 100°C; temperature increasing rate: 200°C/hour; filtering period: 2 seconds; and feedback mode: low. As a reference solution, CBS was used. As a DSC measurement device, VP-Capillary DSC Platform manufactured by MicroCal, Inc. (US) (currently GE Healthcare Bio-Sciences Co., Ltd.) was used for all measurements. Baseline correction was carried out by subtracting the baseline (a scanning curve obtained by also filling a sample cell with the reference solution) from a scanning curve obtained from the sample solution. The value of the peak top temperature in the whole thermogram was defined

as the thermal denaturation midpoint Tm of the Fab region. The results of DSC measurement of 4 types of humanized DR5 antibodies are shown in Fig. 13.

10)-3 In vitro cytocidal activity of CDR-modified hB273 antibody against human cancer cell line

AffiniPure F(ab')$_2$ fragment goat anti-human IgG Fc fragment specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #109-006-098) prepared at 50 μg/ml with 50 mM Tris-HCl (pH 8.5) was dispensed at 45 μL/well in a 96-well microplate (manufactured by Corning Incorporated), and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, the culture supernatant of 293F which was allowed to produce the antibody in 9)-2-1 was added at 50 μL/well such that the final concentration of the antibody was from 150 to 1.5 ng/ml, and the plate was left to stand overnight at 4°C. After each well was washed twice with PBS, Jurkat cells prepared at 4.0 x 10^4 cells/ml in RPMI 1640 medium containing 10% FBS were added at 50 μl/well and cultured under the conditions of 37°C and 5% CO_2 for 23 hours. The amount of ATP derived from viable cells was quantitatively determined using a CellTiter-Glo luminescent cell viability assay kit (manufactured by Promega Corporation, #G7571), and the cytocidal effect of each of the hB273 antibodies was evaluated by taking the value obtained from a well to which the medium

was added in place of the antibody solution as 100%. The cytocidal activity of each of the 4 types of CDR-modified antibodies is shown in Fig. 14.

10)-4 Caspase-3/7 activation effect and in vitro cytocidal activity of hB273_H2-1-NE/L1-NK antibody on human cancer cell lines

Each of a human colon cancer cell line HCT-15 and a human glioblastoma cell line U-87MG was prepared at 1.1×10^5 cells/ml in RPMI 1640 medium containing 10% FBS or MEM (Minimum Essential Medium) medium containing 10% FBS and added to a white clear bottom 96-well microplate (manufactured by Corning Incorporated) at 45 μl/well, and cultured overnight under the conditions of 37°C and 5% CO_2. The hB273_H2-1-NE/L1-NK antibody, the cB273 antibody, or human IgG (manufactured by Jackson ImmunoResearch Laboratories, Inc.) was mixed with the same concentration of AffiniPure goat anti-human IgG Fcγ fragment specific (manufactured by Jackson ImmunoResearch Laboratories, Inc., #109-005-098), and the resulting mixture was added at 5 μl/well such that the final concentration of the hB273_H2-1-NE/L1-NK antibody, the cB273 antibody, or human IgG was 10,000 to 0.1 ng/ml, and the cells were cultured under the conditions of 37°C and 5% CO_2 for 4 hours. The caspase-3/7 activity in each well was measured by a luminometer (manufactured by Perkin Elmer, Inc.) using a Caspase-Glo 3/7

Assay kit (manufactured by Promega Corporation, #G8093). The measurement was performed after incubation at room temperature for 30 minutes according to the protocol attached to the kit. Then, the caspase-3/7 activity was evaluated by taking the value obtained from a well to which the medium was added in place of the antibody solution as 100%. Further, the in vitro cytocidal activity was evaluated by measuring the amount of ATP at 24 hours after treatment with the antibody according to the method shown in Example 3-3. As a result, it was found that the hB273_H2-1-NE/L1-NK antibody has a caspase-3/7 activation effect and a cytocidal effect comparable to those of the cB273 antibody (Fig. 15).

[Example 11] In vivo antitumor effect of cB273 antibody

11)-1 Antitumor activity of cB273 antibody

11)-1-1 Antitumor activity in nude mice implanted with human colon cancer cell line COLO 205

2×10^6 cells of a human colon cancer cell line COLO 205 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice (CAnN.Cg-Foxn1^{nu}/CrlCrlj, purchased from Charles River Laboratories Japan, Inc.). On days 7, 14, and 21 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 1, 3, or 10 mg/kg (n = 10). The major axis and the minor axis of the implanted tumor were measured twice a

week using an electronic digital caliper (manufactured by Mitutoyo Corporation), and the tumor volume was calculated according to the following calculation formula.

Tumor volume (mm^3) = 1/2 x (Minor axis)^2 (mm) x (Major axis)^2 (mm)

The results are shown in Fig. 16. The complete degeneration of the tumor was observed in all mice in the cB273 antibody administration group.

11)-1-2 Antitumor activity in nude mice implanted with human pancreatic cancer cell line MIAPaCa-2

3 x 10^6 cells of a human pancreatic cancer cell line MIAPaCa-2 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 11, 19, 26, and 33 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 3 or 10 mg/kg (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 17. The tumor growth inhibitory rate on day 39 after the implantation, which was the final day of the measurement, was 73.5% in the 3 mg/kg administration group and 77.4% in the 10 mg/kg administration group.

11)-1-3 Antitumor activity in nude mice implanted with human glioblastoma cell line U-87MG

5×10^6 cells of a human glioblastoma cell line U-87MG (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 4, 11, 18, and 25 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 1, 3, or 10 mg/kg (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 18. The tumor growth inhibitory rate on day 32 after the implantation, which was the final day of the measurement, was 99.7% in the 1 mg/kg administration group, 97.8% in the 3 mg/kg administration group, and 98.0% in the 10 mg/kg administration group. Further, the complete degeneration of the tumor was observed in 2 out of 10 mice in the 1 mg/kg administration group, 5 out of 10 mice in the 3 mg/kg administration group, and 3 out of 10 mice in the 10 mg/kg administration group.

11)-1-4 Antitumor activity in nude mice implanted with human lung cancer cell line NCI-H2122 (in combination with paclitaxel and carboplatin)

5×10^6 cells of a human lung cancer cell line NCI-H2122 (purchased from ATCC) were implanted subcutaneously in the

axillary region of nude mice. On days 13 and 20 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 10 mg/kg. Paclitaxel was subcutaneously administered at a dose of 6.25 mg/kg on days 13, 14, 15, 16, and 17 after the implantation. Carboplatin was intraperitoneally administered at a dose of 100 mg/kg on day 13 after the implantation (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 19. The tumor growth inhibitory rate on day 41 after the implantation, which was the final day of the measurement, was 99.6% in the cB273 antibody administration group, 10.2% in the group of combined administration of paclitaxel and carboplatin, and 99.7% in the group of combined administration of the cB273 antibody, paclitaxel, and carboplatin.

11)-1-5 Antitumor activity in nude mice implanted with human lung cancer cell line NCI-H460 (in combination with paclitaxel and carboplatin)

5×10^6 cells of a human lung cancer cell line NCI-H460 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On day 6 after the implantation, the cB273 antibody was intraperitoneally administered to

tumor-bearing mice at a dose of 10 mg/kg. Paclitaxel was subcutaneously administered at a dose of 6.25 mg/kg on days 6, 7, 8, 9, and 10 after the implantation. Carboplatin was intraperitoneally administered at a dose of 100 mg/kg on day 6 after the implantation (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 20. The tumor growth inhibitory rate on day 16 after the implantation, which was the final day of the measurement, was 43.3% in the cB273 antibody administration group, 66.4% in the group of combined administration of paclitaxel and carboplatin, and 79.6% in the group of combined administration of the cB273 antibody, paclitaxel, and carboplatin.

11)-1-6 Antitumor activity in nude mice implanted with human colon cancer cell line DLD-1 (in combination with CPT-11)

5×10^6 cells of a human colon cancer cell line DLD-1 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On day 35 after the implantation, the cB273 antibody was intraperitoneally administered to tumor-bearing mice at a dose of 10 mg/kg. CPT-11 was intraperitoneally administered at a dose of 80 mg/kg on days 35, 40, and 43 after the implantation (n = 10). In the same

manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 21. The tumor growth inhibitory rate on day 55 after the implantation, which was the final day of the measurement, was 25.9% in the cB273 antibody administration group, 29.5% in the CPT-11 administration group, and 72.7% in the group of combined administration of the cB273 antibody and CPT-11.

11)-1-7 Antitumor activity in nude mice implanted with human colon cancer cell line HCT-15 (in combination with CPT-11)

5×10^6 cells of a human colon cancer cell line HCT-15 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On day 7 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 10 mg/kg. CPT-11 was intraperitoneally administered at a dose of 80 mg/kg on days 7, 10, and 14 after the implantation (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 22. The tumor growth inhibitory rate on day 31 after the implantation, which was the final day of the measurement, was 52.8% in the cB273

antibody administration group, 83.5% in the CPT-11 administration group, and 97.8% in the group of combined administration of the cB273 antibody and CPT-11.

11)-1-8 Antitumor activity in nude mice implanted with human colon cancer cell line HCT-116 (in combination with CPT-11)

1×10^7 cells of a human colon cancer cell line HCT-116 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On day 7 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 10 mg/kg. CPT-11 was intraperitoneally administered at a dose of 65 mg/kg on days 7, 10, and 14 after the implantation (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 23. The tumor growth inhibitory rate on day 28 after the implantation, which was the final day of the measurement, was 13.9% in the cB273 antibody administration group, 89.8% in the CPT-11 administration group, and 99.7% in the group of combined administration of the cB273 antibody and CPT-11.

11)-1-9 Antitumor activity in nude mice implanted with human melanoma cell line A375 (in combination with vinblastine)

2×10^6 cells of a human melanoma cell line A375 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 10, 17, and 24 after the implantation, the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 10 mg/kg. Vinblastine was administered through the tail vein at a dose of 10 mg/kg on 10 days after the implantation (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 24. The tumor growth inhibitory rate on day 46 after the implantation, which was the final day of the measurement, was 53.1% in the cB273 antibody administration group, 43.6% in the vinblastine administration group, and 100% in the group of combined administration of the cB273 antibody and vinblastine, and the complete degeneration of the tumor was observed in all mice in the group of combined administration of the cB273 antibody and vinblastine.

11)-2 Comparison of antitumor activity between the cB273 antibody and conatumumab

11)-2-1 Preparation of conatumumab

Conatumumab was prepared based on the amino acid sequences of the light and heavy chains described in WHO Drug

Information, Vol. 22, No. 2, 2008, pp. 129-130.

11)-2-1-1 Construction of conatumumab light chain expression vector

A DNA containing a gene encoding a conatumumab light chain variable region represented by amino acid numbers 21 to 130 of SEQ ID NO: 76 was synthesized (GENEART, Inc. Artificial Gene Synthesis Service). Then, a DNA fragment obtained by cleaving the synthesized DNA with the restriction enzymes NdeI and BsiWI was inserted into the universal humanized antibody light chain expression vector (pEF6KCL) at the site cleaved with the restriction enzymes NdeI and BsiWI, whereby a conatumumab light chain expression vector was constructed. The thus obtained expression vector was named “pEF6KCL/Conatumumab_L”.

11)-2-1-2 Construction of conatumumab heavy chain expression vector

A DNA containing a gene encoding a conatumumab heavy chain variable region represented by amino acid numbers 20 to 141 of SEQ ID NO: 78 in the Sequence Listing was synthesized (GENEART, Inc. Artificial Gene Synthesis Service). Then, a DNA fragment obtained by cleaving the synthesized DNA with the restriction enzyme BlpI was inserted into the universal humanized antibody heavy chain expression vector (pEF1FCCU)

at the site cleaved with the restriction enzyme BlpI, whereby a conatumumab heavy chain expression vector was constructed. The thus obtained expression vector was named “pEF1FCCU/Conatumumab_H”.

11)-2-1-3 Production of conatumumab

1.2×10^9 cells of FreeStyle 293F cells (Invitrogen Corporation) in the logarithmic growth phase were seeded into 1.2 L of fresh FreeStyle 293 Expression Medium (Invitrogen Corporation) and cultured for 1 hour by shaking at 90 rpm at 37°C in an 8% CO_2 incubator. 3.6 mg of polyethyleneimine (Polyscience #24765) was dissolved in 20 ml of Opti-Pro SFM medium (Invitrogen Corporation). Subsequently, the heavy chain expression vector pEF1FCCU/Conatumumab_H (0.4 mg) and the light chain expression vector pEF6KCL/Conatumumab_L (0.8 mg) prepared with PureLink HiPure Plasmid Kit (Invitrogen Corporation) were suspended in 20 ml of Opti-Pro SFM medium. Then, 20 ml of the obtained expression vectors/Opti-Pro SFM mixed liquid was added to 20 ml of the obtained polyethyleneimine/Opti-Pro SFM mixed liquid, and the resulting mixture was gently stirred and then left for 5 minutes. Thereafter, the mixture was added to the FreeStyle 293F cells, and culture shaking at 90 rpm was performed for 7 days at 37°C in an 8% CO_2 incubator. The resulting culture supernatant was filtered through a disposable capsule filter (Advantec

#CCS-045-E1H).

11)-2-1-4 Purification of conatumumab

The culture supernatant obtained in the above 11)-2-1-3 was purified by a two-step process including rProtein A affinity chromatography (at 4 to 6°C) and ceramic hydroxyapatite (at room temperature). A buffer replacement step after the purification by rProtein A affinity chromatography and after the purification by ceramic hydroxyapatite was performed at room temperature. First, 1100 to 1200 ml of the culture supernatant was applied to MabSelect SuRe (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap columns (volume: 1 ml) connected in series) equilibrated with PBS. After all the culture solution was poured into the column, the column was washed with 15 to 30 ml of PBS. Subsequently, elution was performed with a 2 M arginine hydrochloride solution (pH 4.0), and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the buffer was replaced with a buffer containing 5 mM sodium phosphate, 50 mM MES, and 20 mM NaCl at pH 6.5. Further, the antibody solution subjected to buffer replacement was applied to a ceramic hydroxyapatite column (Japan Bio-Rad Laboratories, Inc., Bio-Scale CHT2-1

hydroxyapatite column (volume: 2 ml)) equilibrated with a buffer containing 5 mM NaPi, 50 mM MES, and 20 mM NaCl at pH 6.5. Then, linear concentration gradient elution with sodium chloride was performed, and a fraction containing the antibody was collected. The fraction was applied to a desalting column (manufactured by GE Healthcare Bio-Sciences Co., Ltd., two HiTrap desalting columns (volume: 5 ml) connected in series), whereby the liquid was replaced with CBS (10 mM citrate buffer containing 140 mM sodium chloride, pH 6.0). Finally, the resulting solution was concentrated using Centrifugal UF Filter Device VIVASPIN 20 (fractional molecular weight: 30 K, Sartorius Co., Ltd., at 4°C), and the concentration of IgG was adjusted to 1.0 mg/ml or more, and the thus obtained solution was used as a purified sample.

11)-2-2 Comparison of antitumor activity in nude mice implanted with human colon cancer cell line HCT-15 between the cB273 antibody and conatumumab

1×10^7 cells of a human colon cancer cell line HCT-15 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 6, 13, and 20 after the implantation, the cB273 antibody or conatumumab was administered to tumor-bearing mice through the tail vein at a dose of 3, 10, or 30 mg/kg (n = 10). In the same manner as described above, the major axis and the minor axis of the

implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 25. The tumor growth inhibitory rate on day 30 after the implantation, which was the final day of the measurement, was 57.9% in the group administered the cB273 antibody at 3 mg/kg, 56.0% in the group administered the cB273 antibody at 10 mg/kg, 53.6% in the group administered the cB273 antibody at 30 mg/kg, 27.4% in the group administered conatumumab at 3 mg/kg, 26.9% in the group administered conatumumab at 10 mg/kg, and 20.3% in the group administered conatumumab at 30 mg/kg.

11)-2-3 Comparison of antitumor activity in nude mice implanted with human lung cancer cell line NCI-H1975 between the cB273 antibody and conatumumab

3×10^6 cells of a human lung cancer cell line NCI-H1975 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 12, 19, and 26 after the implantation, the cB273 antibody or conatumumab was administered to tumor-bearing mice through the tail vein at a dose of 3 or 10 mg/kg (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 26. The tumor growth inhibitory rate on day 32 after the implantation, which was

the final day of the measurement, was 71.5% in the group administered the cB273 antibody at 3 mg/kg, 73.3% in the group administered the cB273 antibody at 10 mg/kg, 13.5% in the group administered conatumumab at 3 mg/kg, and 12.6% in the group administered conatumumab at 10 mg/kg.

[Example 12] In vivo antitumor effect of hB273_H2-1-NE/hB273_L1-NK antibody

12)-1 Antitumor activity of hB273_H2-1-NE/hB273_L1-NK antibody in nude mice implanted with human colon cancer cell line COLO 205

2×10^6 cells of a human colon cancer cell line COLO 205 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On days 8, 15, and 22 after the implantation, the hB273_H2-1-NE/hB273_L1-NK antibody or the cB273 antibody was administered to tumor-bearing mice through the tail vein at a dose of 0.3 or 3 mg/kg (n = 10). In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 27. The complete degeneration of the tumor was observed in all mice in the group administered the hB273_H2-1-NE/hB273_L1-NK antibody at 3 mg/kg and the group administered the cB273 antibody at 0.3 mg/kg. Further, the complete degeneration of the tumor was observed

in 9 out of 10 mice in the group administered the hB273_H2-1-NE/hB273_L1-NK antibody at 0.3 mg/kg, and 8 out of 10 mice in the group administered the cB273 antibody at 3 mg/kg.

[Example 13] In vitro cytocidal activity of hB273_H2-1-NE/hB273_L1-NK antibody against human cancer cell lines

NCI-N87, KATO-III, and SNU-16 (each of which is a human stomach cancer cell line), Caki-1, ACHN, and 786-O (each of which is a human renal cancer cell line), Hep3B, SK-HEP-1, and HepG2 (C3A) (each of which is a human liver cancer cell line), and HT-1080 (which is a human fibrosarcoma cell line) were purchased from American Type Culture Collection (ATCC). GCIY (which is a human stomach cancer cell line) was purchased from RIKEN. HuH-7 (which is a human liver cancer cell line) was purchased from National Institute of Biomedical Innovation.

The in vitro cytocidal activities against various types of cell lines were measured by the following method. As for the stomach cancer cell line, the renal cancer cell line, and the fibrosarcoma cell line, appropriately subcultured cells were counted by a trypan blue staining method, and thereafter prepared at 1 x 10^5 cells/ml in a medium containing 10% fetal bovine serum (manufactured by HyClone Laboratories, Inc.) (hereinafter referred to as “the medium”). In the medium, the hB273_H2-1-NE/hB273_L1-NK antibody at 20 μg/ml and a secondary antibody (goat anti-human IgG antibody, manufactured by MP

Biomedicals, LLC.) at 40 μg/ml were mixed. Then, the resulting mixture was diluted with the medium, whereby solutions were prepared such that the concentration of the hB273_H2-1-NE/hB273_L1-NK antibody was 2000, 200, 20, or 2 ng/ml. Each of the resulting solutions having the respective concentrations was added to a transparent 96-well microplate (manufactured by Corning Incorporated) at 50 μl/well (3 wells per group), and the cell suspension was seeded at 50 μl/well (5×10^3 cells) (the final concentration of the hB273_H2-1-NE/hB273_L1-NK antibody: 10000, 1000, 100, 10, or 1 ng/ml).

As for the liver cancer cell line, appropriately subcultured cells were counted by a trypan blue staining method, and thereafter prepared at 4×10^4 cells/ml in the medium. In the medium, the hB273_H2-1-NE/hB273_L1-NK antibody at 2 μg/ml and the secondary antibody at 4 μg/ml were mixed. Then, the resulting mixture was diluted with the medium, whereby solutions were prepared such that the concentration of the hB273_H2-1-NE/hB273_L1-NK antibody was 200, 20, 2, 0.2, or 0.02 ng/ml. Each of the resulting solutions having the respective concentrations was added to a black clear bottom 96-well microplate (manufactured by Corning Incorporated) at 50 μl/well (2 wells per group), and the cell suspension was seeded at 50 μl/well (2×10^3 cells) (the final concentration of the antibody: 1000, 100, 10, 1, 0.1, or 0.01 ng/ml).

The cells were cultured at 37°C in the presence of 5% CO_2 for 72 hours, and the amount of ATP in each well was measured. The measurement of the amount of ATP was performed using a luciferase luminescent reagent (CellTiter-Glo, manufactured by Promega Corporation) according to the attached protocol. That is, a test solution composed of a cell lysate component and a luminescent substrate component was added to the plate at 100 μl per well, followed by stirring. Thereafter, the luminescence from each well was measured using a luminometer (manufactured by Berthold Technologies). As for the stomach cancer cell line, the renal cancer cell line, and the fibrosarcoma cell line, a test solution in an amount of 100 μl per well was transferred from the transparent 96-well microplate to a white 96-well microplate (manufactured by Corning Incorporated), and then, the luminescence was measured.

A well to which the medium and the cell suspension were added was prepared as a negative control well, a well to which only the medium was added was prepared as a background well, and the cell viability in each test well was calculated according to the following formula.

Cell viability (%) = (Luminescence intensity of test well - Average luminescence intensity of background well) / (Average luminescence intensity of negative control well - Average luminescence intensity of background well) x 100

In Fig. 51, an average of the cell viability of each cell line for the respective concentrations of the antibody used in the treatment is shown. As for the stomach cancer cell line, the renal cancer cell line, and the fibrosarcoma cell line, a standard error is represented by an error bar. The hB273_H2-1-NE/hB273_L1-NK antibody exhibited a cytotoxic activity against all of the cell lines tested except for 786-O.

[Example 14] Measurement of in vivo activity of hB273_H2-1-NE/hB273_L1-NK antibody in combination with a chemotherapeutic agent

14)-1 Antitumor activity of hB273_H2-1-NE/hB273_L1-NK antibody in combination with 5-FU in nude mice implanted with human colon cancer cell line HCT-15 and comparison of activity with conatumumab

1×10^7 cells of a human colon cancer cell line HCT-15 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice (CAnN.Cg-Foxn1^{nu}/CrlCrlj, purchased from Charles River Laboratories Japan, Inc.). On days 7, 14, and 21 after the implantation, the hB273_H2-1-NE/hB273_L1-NK antibody or conatumumab was administered to tumor-bearing mice through the tail vein at a dose of 3 mg/kg. 5-FU was administered through the tail vein on day 7 after the implantation at a dose of 160 mg/kg. The experiment was carried out at n = 6. The major axis and the minor axis of the implanted

tumor were measured twice a week using an electronic digital caliper (manufactured by Mitutoyo Corporation), and the tumor volume was calculated according to the following calculation formula.

Tumor volume (mm^3) = 1/2 x (Minor axis)^2 (mm) x (Major axis)^2 (mm)

The results are shown in Fig. 52. The tumor growth inhibitory rate on day 28 after the implantation, which was the final day of the measurement, was 62% in the hB273_H2-1-NE/hB273_L1-NK antibody administration group, 27% in the conatumumab administration group, 54% in the 5-FU administration group, 91% in the group of combined administration of the hB273_H2-1-NE/hB273_L1-NK antibody and 5-FU, and 78% in the group of combined administration of conatumumab and 5-FU. That is, a combined effect of the hB273_H2-1-NE/hB273_L1-NK antibody and 5-FU was observed, and further, a higher antitumor activity was observed in the group of combined administration of the hB273_H2-1-NE/ hB273_L1-NK antibody and 5-FU than in the group of combined administration of conatumumab and 5-FU.

14)-2 Antitumor activity of hB273_H2-1-NE/hB273_L1-NK antibody in combination with paclitaxel in nude mice implanted with human non-small cell lung cancer cell line NCI-H1975 and comparison of activity with conatumumab

3×10^6 cells of a human non-small cell lung cancer cell line NCI-H1975 (purchased from ATCC) were implanted subcutaneously in the axillary region of nude mice. On day 11, 18, and 25 after the implantation, the hB273_H2-1-NE/hB273_L1-NK antibody or conatumumab was administered to tumor-bearing mice through the tail vein at a dose of 3 mg/kg. Paclitaxel was administered to tumor-bearing mice through the tail vein on days 11, 12, 13, and 14 after the implantation at a dose of 6.25 mg/kg. The experiment was carried out at n = 6. In the same manner as described above, the major axis and the minor axis of the implanted tumor were measured, and the tumor volume was calculated.

The results are shown in Fig. 53. The tumor growth inhibitory rate on day 32 after the implantation, which was the final day of the measurement, was 66% in the hB273_H2-1-NE/hB273_L1-NK antibody administration group, 40% in the conatumumab administration group, 49% in the paclitaxel administration group, 91% in the group of combined administration of the hB273_H2-1-NE/hB273_L1-NK antibody and paclitaxel, and 79% in the group of combined administration of conatumumab and paclitaxel. That is, a combined effect of the hB273_H2-1-NE/hB273_L1-NK antibody and paclitaxel was observed, and further, a higher antitumor activity was observed in the group of combined administration of the hB273_H2-1-NE/hB273_L1-NK antibody and paclitaxel than in the

group of combined administration of conatumumab and paclitaxel.

Claims

[Claim 1]

An antibody capable of binding to DR5, characterized in that:

the heavy chain sequence contains a variable region having CDRH1, CDRH2, and CDRH3, and the CDRH1 comprises an amino acid sequence represented by SEQ ID NO: 82, the CDRH2 comprises either one of the amino acid sequences represented by SEQ ID NOS: 89 and 83, and the CDRH3 comprises an amino acid sequence represented by SEQ ID NO: 84; and

the light chain sequence contains a variable region having CDRL1, CDRL2, and CDRL3, and the CDRL1 comprises any one of the amino acid sequences represented by SEQ ID NOS: 87, 85, 86, 88 and 79, the CDRL2 comprises an amino acid sequence represented by SEQ ID NO: 80, and the CDRL3 comprises an amino acid sequence represented by SEQ ID NO: 81, or a functional fragment of the antibody having antigen-binding activity.

[Claim 2]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 1, characterized by containing a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 20 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 16.

[Claim 3]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 1 or 2, characterized in that the antibody is a chimeric antibody.

[Claim 4]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 3, characterized by containing a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 20 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 16.

[Claim 5]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 1, characterized in that the antibody is humanized.

[Claim 6]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 5, characterized by containing:

(a) a heavy chain variable region sequence selected from the group consisting of the following amino acid sequences:

a1) an amino acid sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70;

a2) an amino acid sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 42;

a3) an amino acid sequence having a homology of at least 95% with an amino acid sequence selected from a1) and a2);

a4) an amino acid sequence having a homology of at least 99% with an amino acid sequence selected from a1) and a2); and

a5) an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in either one of the amino acid sequences selected from a1) and a2); and

(b) a light chain variable region sequence selected from the group consisting of the following amino acid sequences:

b1) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 62;

b2) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 52;

b3) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 58;

b4) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 66;

b5) an amino acid sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 28;

b6) an amino acid sequence having a homology of at least 95% with any one of the amino acid sequences selected from b1) to b5);

b7) an amino acid sequence having a homology of at least 99% with any one of the amino acid sequences selected from b1) to b5); and

b8) an amino acid sequence including a substitution, deletion, or addition of one to several amino acid residues in any one of the amino acid sequences selected from b1) to b5).

[Claim 7]

The antibody or a functional fragment of the antibody having antigen-binding activity according to claim 6, characterized by containing: i) a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 62; ii) a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid

sequence represented by SEQ ID NO: 52;iii) a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 58;iv) a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 42 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 28; v) a heavy chain variable region sequence comprising amino acid residues 20 to 141 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain variable region sequence comprising amino acid residues 21 to 134 of the amino acid sequence represented by SEQ ID NO: 66; vi) a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 62; vii) a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 52;viii) a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino

acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 58;ix) a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 42 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 28; or x) a heavy chain sequence comprising amino acid residues 20 to 471 of the amino acid sequence represented by SEQ ID NO: 70 and a light chain sequence comprising amino acid residues 21 to 239 of the amino acid sequence represented by SEQ ID NO: 66.

[Claim 8]

The functional fragment of the antibody having antigen-binding activity according to any one of claims 1 to 7, which is selected from the group consisting of Fab, F(ab')2, Fab' and Fv.

[Claim 9]

A pharmaceutical composition characterized by comprising at least one of the antibodies or functional fragments of the antibodies having antigen-binding activity according to claims 1 to 8.

[Claim 10]

The pharmaceutical composition according to claim 9, characterized by being a pharmaceutical composition for treating and/or preventing cancer.

[Claim 11]

A pharmaceutical composition for treating and/or preventing cancer, characterized by comprising at least one of the antibodies or functional fragments of the antibodies having antigen-binding activity according to claims 1 to 8 and at least one member selected from the group consisting of paclitaxel, carboplatin, CPT-11, and vinblastine.

[Claim 12]

The pharmaceutical composition according to claim 10 or 11, wherein the cancer is selected from the group consisting of lung cancer, prostate cancer, thyroid cancer, stomach cancer, liver cancer, ovarian cancer, colon cancer, breast cancer, pancreatic cancer, uterine cancer, melanoma, glioblastoma, and blood cell cancer.

[Claim 13]

An antibody or functional fragment thereof having antigen-binding activity according to any one of claims 1 to 8 for use in the treatment and/or prevention of cancer.

[Claim 14]

An antibody or functional fragment thereof having antigen-binding activity according to any one of claims 1 to 8 for use in the treatment and/or prevention of cancer, characterized by simultaneously or sequentially administering the antibody or functional fragment having antigen-binding activity and at least one member selected from the group consisting of paclitaxel, carboplatin, CPT-11, vinblastine,

and 5-FU.

[Claim 15]

A product selected from the group consisting of paclitaxel, carboplatin, CPT-11, vinblastine, and 5-FU for use in the treatment and/or prevention of cancer characterized by simultaneously or sequentially administering an antibody or functional fragment thereof having antigen-binding activity according to any one of claims 1 to 8.

[Claim 16]

The antibody or product for use according to any one of claims 13 to 15, wherein the cancer is selected from the group consisting of lung cancer, prostate cancer, thyroid cancer, stomach cancer, liver cancer, ovarian cancer, colon cancer, breast cancer, pancreatic cancer, renal cancer, uterine cancer, melanoma, fibrosarcoma, glioblastoma, and blood cell cancer.

[Claim 17]

A polynucleotide encoding the antibody according to any one of claims 2, 4, 6 or 7.

[Claim 18]

The polynucleotide according to claim 17, characterized by containing: i) a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 19 and a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 15; or ii) a nucleotide sequence comprising nucleotides 58 to 1413

of the nucleotide sequence represented by SEQ ID NO: 19 and a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 15.

[Claim 19]

The polynucleotide according to claim 17, characterized by containing:

(a) a polynucleotide selected from the group consisting of the following nucleotide sequences:

a1) a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69;

a2) a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 41;

a3) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to a nucleotide sequence selected from a1) and a2) under stringent conditions; and

a4) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in a nucleotide sequence selected from a1) and a2); and

(b) a polynucleotide selected from the group consisting of the following nucleotide sequences:

b1) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 61;

b2) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 51;

b3) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 57;

b4) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 65;

b5) a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 27;

b6) a nucleotide sequence of a polynucleotide which hybridizes to a polynucleotide comprising a nucleotide sequence complementary to any one of the nucleotide sequences selected from b1) to b5) under stringent conditions; and

b7) a nucleotide sequence including a substitution, deletion, or addition of one to several nucleotides in any one of the nucleotide sequences selected from b1) to b5).

[Claim 20]

The polynucleotide according to claim 19, characterized by containing:

i) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 61; ii) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide

sequence represented by SEQ ID NO: 51; iii) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 57; iv) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 41, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 27; v) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 423 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 402 of the nucleotide sequence represented by SEQ ID NO: 65; vi) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 61; vii) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 51; viii) a polynucleotide

comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 57 ix) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 41, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 27; or x) a polynucleotide comprising a nucleotide sequence comprising nucleotides 58 to 1413 of the nucleotide sequence represented by SEQ ID NO: 69, and a polynucleotide comprising a nucleotide sequence comprising nucleotides 61 to 717 of the nucleotide sequence represented by SEQ ID NO: 65.

[Claim 21]

A vector comprising any one of the polynucleotides according to claims 17 to 20.

[Claim 22]

A transformed host cell comprising any one of the polynucleotides according to claims 17 to 20, or the vector according to claim 21.

[Claim 23]

A method of producing the antibody according to any one of claims 2, 4, 6 or 7, comprising the step of culturing the host cell according to claim 22 and purifying the antibody from

the resulting cultured product.