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(54) Titre : ANTICORPS ANTI-A33
(54) Title: ANTI-A33 ANTIBODY

(57) **Abrégé/Abstract:**

The present invention provides: an antibody or a antibody fragment thereof, which can bind to A33, which specifically attacks A33-expressing tumor cells with the use of ADCC and CDC based on the immune system, and for which no HAHA is produced; and a preventive or therapeutic agent for various malignant tumors including solid tumors that are currently treated with difficulty, which contains the antibody or an antibody fragment thereof. Specifically, the antibody or a functional fragment thereof is capable of binding to A33 and is produced by a hybridoma M10 (accession No. FERM BP-10107), M96 (accession No. FERM BP-10108), M165 (accession No. FERM BP-10106), N26 (accession No. FERM BP-10109), Q47 (accession No. FERM BP-10104), Q54 (accession No. FERM BP-10105), or R5 (accession No. FERM BP-10107). The preventive or therapeutic agent for tumors contains the antibody or a functional fragment thereof.



ABSTRACT

The present invention provides: an antibody or a antibody fragment thereof, which can bind to A33, which specifically attacks A33-expressing tumor cells with the use of ADCC and CDC based on the immune system, and for which no HAHA is produced; and a preventive or therapeutic agent for various malignant tumors including solid tumors that are currently treated with difficulty, which contains the antibody or an antibody fragment thereof. Specifically, the antibody or a functional fragment thereof is capable of binding to A33 and is produced by a hybridoma M10 (accession No. FERM BP-10107), M96 (accession No. FERM BP-10108), M165 (accession No. FERM BP-10106), N26 (accession No. FERM BP-10109), Q47 (accession No. FERM BP-10104), Q54 (accession No. FERM BP-10105), or R5 (accession No. FERM BP-10107). The preventive or therapeutic agent for tumors contains the antibody or a functional fragment thereof.

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DESCRIPTION

ANTI-A33 ANTIBODY

Technical Field

The present invention relates to an anti-A33 antibody that specifically binds to an A33 antigen. Furthermore, the present invention relates to a preventive or therapeutic agent for diseases due to A33-expressing cells, which comprises the anti-A33 antibody as an active ingredient. The present invention particularly relates to a therapeutic agent for malignant tumors.

Background Art

Cancer (tumor) is the leading cause of death in Japan and the number of cancer patients is increasing each year. Hence, the development of drugs or therapeutic methods against cancer that have high effectiveness and safety has been strongly desired. Among various types of cancer, colorectal cancer accounted for 12.2% of total cancer cases, as shown in the survey conducted in 1999. The colorectal cancer mortality rates ranked third in the case of men and second in the case of women. Based on the significantly increased number of colorectal cancer cases in recent years, colorectal cancer morbidity or mortality is predicted to surpass the same for gastric cancer in the future. Furthermore, gastric cancer accounted for 17.4% of total cancer cases, as shown in the survey conducted in 1999, and the mortality rates thereof ranked second in the case of men and first in the case of women.

The use of an antibody as a remedy is being recognized as an important and valuable approach for the treatment of various pathological conditions (cancer types). Antibody specificity is useful for treating pathological conditions wherein a tumor-specific antigen exhibits the properties of heterologous cells. Antibodies

effectively target such cells through binding to tumor-specific antigens, which are proteins to be expressed on cell surfaces. Currently, a chimeric antibody (Rituximab) that targets at CD20 (the receptor existing on the cell membranes), a monoclonal antibody such as a humanized antibody that targets at Her2/neu, and the like have been used against malignant tumors as subject diseases. The therapeutic effects thereof have been recognized. Antibodies are characterized by long blood half-life and high specificity to antigens, and they are particularly useful as antitumor agents. For example, in the case of an antibody that targets a tumor-specific antigen, it is inferred that the administered antibody is accumulated in tumors. Furthermore, the immune system that attacks against cancer cells through the use of complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) can be expected. Moreover, previous binding of a drug (such as a radioactive nuclide or a cytotoxic substance) to the antibody enables efficient delivery of the bound drug to tumor sites. Simultaneously, since the amounts of such drugs that reach other non-specific tissues are decreased, alleviation of side effects can also be expected. Through administration of an antibody having agonistic activity when a tumor-specific antigen has activity of inducing cell death or through administration of an antibody having neutralization activity when a tumor-specific antigen is involved in the growth and survival of cells, the accumulation of such tumor-specific antibody and the suspension of tumor growth or the degeneration of tumors through the activity of such antibody can be expected. It is thought that antibodies are appropriate for use as antitumor agents because of their features as described above.

Mice have been used as target animals in early antibody production. However, the use of mouse antibodies *in vivo* is limited for many reasons. Mouse antibodies that are recognized by human hosts as extraneous matter induce "human anti-mouse antibodies," with such induction referred to as the "HAMA" response (see Schiff et al., Canc. Res. (1985), 45, 879-885). Furthermore, the Fc portions of mouse antibodies are

not effective for stimulation of human complements or cytotoxicity.

A chimeric antibody has been developed as an approach to avoid such problem (see European Patent Application Nos. 120694 and 125023). A chimeric antibody contains parts of antibodies derived from 2 or more species (e.g., a mouse antibody variable region and a human antibody constant region). Such chimeric antibody has the advantage of retaining the features of a mouse antibody and can stimulate a human complement or cytotoxicity because of its human Fc. However, such chimeric antibody still induces a "human anti-chimeric antibody;" that is, "HACA" response (see Bruggemann, et al., J. Exp. Med., 170, 2153-2157, 1989).

Furthermore, a recombinant antibody has been developed, wherein only one substituted antibody portion is a complementarity determining region (that is, "CDR") (British Patent No. GB2188638A and United States Patent No. 5585089). An antibody comprising mouse CDR, a human variable framework, and a constant region (that is, a "humanized antibody") has been produced using CDR-grafting technology (see Riechmann, et al., Nature (1988), 332, 323-327).

A mouse anti-A33 antibody against an antigen that is a class I cell membrane protein referred to as "A33," a member of the Ig superfamily, and a tumor-specific antigen and a humanized antibody have been reported (see United States Patent No. 5958412, Description; King D. J. et al., British J. Cancer (1995) 72, 1364-1372; Welt S. et al., J. Clinical Oncology (1994), 12, 1561-1571; Welt S. et al., J. Clinical Oncology (1996), 14, 1787-1797; Welt S. et al., Clinical Cancer Res. (2003), 9, 1338-1346; and Welt S. et al., Clinical Cancer Res. (2003), 9, 1347-1353). Involvement of such antigen in colon cancer and gastric cancer is known (see United States Patent No. 5643550, Description; United States Patent No. 5160723, Description; and Garin-Chesa P. G. et al., International J. Oncology (1996), 9, 465-471). Furthermore, in recent years, phase I clinical tests using the humanized A33 antibody have been conducted for colon cancer patients (see Welt S. et al., Clinical Cancer Res. (2003), 9, 1338-1346 and Welt S. et al.,

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Clinical Cancer Res. (2003), 9, 1347-1353). In the former report about the administration of the antibody alone, partial reactions were observed in 1 out of 11 patients to which the antibody could be administered. Moreover, in the latter report about the test using the antibody and chemotherapy in combination, partial reactions were observed in 3 out of 12 patients to which the antibody could be administered, and a mixed reaction was observed in 1 of the same. Even in the case of Avastin* (Bevacizumab; humanized anti-VEGF antibody) under development by Genentech in recent years, there is a report that 1 out of 12 patients showed partial reactions in the phase I clinical test using Avastin and standard chemotherapy in combination (Margolin K. et al., J. Clin. Oncol. (2001) 19, 851-856). Accordingly, based on the result that 1 out of 11 patients showed partial reactions as a result of the administration of the antibody alone, the antibody is expected to exert extensive antitumor effects against colorectal cancer.

Although the humanized A33 antibody showed very significant tumor reactions in the phase I clinical tests as described above, human anti-humanized antibodies (that is, "HAHAs") were produced in both tests with probabilities as high as 50% or more. Interestingly, no HAHA was observed in patients in which the antibody showed high tumor reactivity.

Disclosure of the Invention

The object of the present invention is to provide a preventive or therapeutic agent for various malignant tumors, including solid tumors, that are currently treated with difficulty through the development of an antibody that can bind to A33, specifically attacks A33-expressing tumor cells with the use of ADCC or CDC based on the immune system, and does not cause the production of HAHA.

As described above, it is thought that an antibody that targets the A33 antigen is appropriate for use as an antitumor agent. In addition, with the use of such an antibody

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by which no HAHA is produced, even higher antitumor effects may be obtained. Hence, as a result of intensive studies concerning the production of an antibody against A33, the present inventors have succeeded in obtaining a monoclonal antibody showing antitumor effects against A33-expressing cancer cells and have specified the sequences of the variable regions of the monoclonal antibody. Thus, the present inventors have completed the present invention.

Specifically, the present invention is as described below.

In the first aspect, the present invention provides a monoclonal antibody binding to A33, which is produced by a mouse-mouse hybridoma. Specifically, a monoclonal antibody, which is preferably a human antibody, or a functional fragment thereof produced by 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, or 125R5AAAA, for example, is provided. The type of such monoclonal antibody produced by 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, or 125R5AAAA is human immunoglobulin G (IgG) type. Among the above hybridomas, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA were deposited on August 24, 2004, with the International Patent Organism Depositary (IPOD) (Central 6, 1-1, Higashi 1, Tsukuba, Ibaraki, Japan), the National Institute of Advanced Industrial Science and Technology (AIST) under accession Nos. FERM BP-10107 (denotation for identification: M10), FERM BP-10106 (denotation for identification: M165), FERM BP-10108 (denotation for identification: M96), FERM BP-10109 (denotation for identification: N26), FERM BP-10104 (denotation for identification: Q47), FERM BP-10105 (denotation for identification: Q54), and FERM BP-10103 (denotation for identification: R5), respectively.

In an embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof, which has the variable regions of an antibody produced by one of the above hybridomas.

In another embodiment of the present invention, examples of the antibody of the present invention include antibodies of modified subclasses. Specifically, the antibody of the present invention may be: an antibody or a functional fragment thereof which is produced by hybridoma 263A17 and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125M10AA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125M165DAAA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125M96ABA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125N26F6AA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125Q47BA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; an antibody or a functional fragment thereof which is produced by hybridoma 125Q54AAAA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4; or an antibody or a functional fragment thereof which is produced by hybridoma 125R5AAAA and is of the subclass human IgG1, human IgG2, human IgG3, or human IgG4.

Furthermore, in another aspect of the present invention, the present invention provides an antibody or a functional fragment thereof, which binds to A33 and contains the variable regions of an antibody produced by hybridoma 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, or 125R5AAAA.

In an embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 23 and 25. In another embodiment of the

present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 27 and 29. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 31 and 33. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 35 and 37. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 39 and 41. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 43 and 45. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 47 and 49. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the variable regions of the amino acid sequences represented by SEQ ID NOS: 51 and 53. In another embodiment of the present invention, the antibody of the present invention is an antibody or a functional fragment thereof having the entire region of both of the amino acid sequences represented by SEQ ID NOS: 87 and 89.

In another aspect, the present invention further provides the above antibody or a functional fragment thereof that suppresses tumor growth (e.g., derived from colorectal cancer cell line COLO205 cells implanted in nude mice). Upon the suppression of tumors, the antibody or a functional fragment thereof of the present invention is administered to a tumor-bearing animal to be tested (e.g., a tumor-bearing experimental animal such as a colon cancer cell tumor-bearing mouse model having a body weight of

20 g) in an amount between 10 µg/body and 100 µg/body. The dose is 100 µg/body or 5 mg/kg, and preferably 10 µg/body or 0.5 mg/kg, for example.

In an embodiment of the present invention, the antibody of the present invention has one of the following properties.

(a) ADCC test

In the presence of normal human peripheral blood mononuclear cells, the antibody of the present invention exerts antibody-dependent cellular cytotoxicity (ADCC) against A33-expressing human cancer cells.

(b) CDC test

In the presence of human serum-derived complements, the antibody of the present invention exerts complement-dependent cytotoxicity (CDC) against A33-expressing human cancer cells.

(c) *In vivo* test

The antibody of the present invention exerts antitumor effects against non-human animals bearing A33-expressing human cancer cells.

(d) Competition test

The antibody of the present invention is (i) strongly competitive (blocker), (ii) weakly competitive (partial blocker), or (iii) not competitive (non-blocker) with chimeric anti-A33 (comprising the heavy chain variable region and the light chain variable region of an antibody produced by hybridoma ATCC HB-8779 and the heavy chain constant region and the light chain constant region of human IgG1).

(e) Immunohistochemical test

The presence of the antibody of the present invention results in the staining of human adult colon cancer tissues, human adult normal colon tissues, and human normal small intestine tissues.

In another aspect, the present invention further provides: a nucleic acid encoding an antibody possessed by a hybridoma selected from the group consisting of hybridomas

125M10AA (accession No. FERM BP-10107), 125M165DAAA (accession No. FERM BP-10106), 125M96ABA (accession No. FERM BP-10108), 125N26F6AA (accession No. FERM BP-10109), 125Q47BA (accession No. FERM BP-10104), 125Q54AAAA (accession No. FERM BP-10105), and 125R5AAAA (accession No. FERM BP-10103) or a nucleic acid encoding a functional fragment of the antibody; a protein encoded by the nucleic acid; an expression vector having the above nucleic acid; and a host selected from the group consisting of *Escherichia coli*, a yeast cell, an insect cell, a mammalian cell, a plant cell, and a mammal having the expression vector.

In another aspect, the present invention further provides a method for producing an anti-A33 monoclonal antibody or a functional fragment thereof, which comprises: isolating a gene encoding an anti-A33 monoclonal antibody (e.g., a gene encoding a variable region of a heavy chain amino acid sequence and a gene encoding a variable region of a light chain amino acid sequence) from a hybridoma selected from the group consisting of hybridomas 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA; constructing an expression vector having such gene; introducing the expression vector into a host; culturing the host; causing the expression of the monoclonal antibody; and harvesting the anti-A33 monoclonal antibody or a functional fragment thereof from the thus obtained host or culture products such as the culture supernatant of the host, the secretion product of the host, or the like.

In another aspect, the present invention further provides a preventive, therapeutic, or diagnostic agent for tumors, which contains the above antibody or a functional fragment thereof as an active ingredient.

Examples of tumors that can be prevented or treated with the use of the above agent include at least one tumor selected from the group consisting of colorectal cancer, colon cancer, rectal cancer, gastric cancer, pancreatic cancer, breast cancer, melanoma, renal cell cancer, cervical cancer, endometrial cancer, ovarian cancer, esophageal cancer,

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prostatic cancer, testicular cancer, and mesothelial cancer.

In another embodiment of the present invention, the antibody or a functional fragment thereof of the present invention is characterized in that a dose of 10 µg/body or 100 µg/body of the above antibody or a functional fragment thereof is confirmed to exhibit suppression (after tumor grafting) of the tumors of tumor-bearing nude mice wherein COLO205 cells are implanted to a more significant degree than that in the case of a group of the mice to which a vehicle is administered or a group of the mice to which an anti-DNP-IgG1 antibody is administered.

10 The present invention also relates to: an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma M10 (accession No. FERM BP-10107); an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma M96 (accession No. FERM BP-10108); an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma M165 (accession No. FERM BP-10106); an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma N26 (accession No. FERM BP-10109); an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma Q47 (accession No. FERM BP-10104); an antibody binding to A33, which
20 recognizes the same epitope as that recognized by an antibody that is produced by hybridoma Q54 (accession No. FERM BP-10105); and an antibody binding to A33, which recognizes the same epitope as that recognized by an antibody that is produced by hybridoma R5 (accession No. FERM BP-10103).

This description includes part or all of the contents as disclosed in the description and/or drawings of Japanese Patent Application No. 2004-259090, which is a priority document of the present application.

Brief Description of the Drawings

Fig. 1A shows ADCC activity determined when COLO205 cells were targeted using each purified monoclonal antibody.

Fig. 1B shows CDC activity determined when COLO205 cells were targeted using each purified monoclonal antibody.

Fig. 1C shows ADCC activity determined when NCI-H508 cells were targeted using each purified monoclonal antibody.

Fig. 1D shows CDC activity determined when NCI-H508 cells were targeted using each purified monoclonal antibody.

Fig. 2A shows ADCC activity determined when COLO205 cells were targeted using recombinant antibodies.

Fig. 2B shows CDC activity determined when COLO205 cells were targeted using recombinant antibodies.

Fig. 2C shows ADCC activity determined when NCI-H508 cells were targeted using recombinant antibodies.

Fig. 2D shows CDC activity determined when NCI-H508 cells were targeted using recombinant antibodies.

Fig. 3A is a photograph showing the results of Western blot analysis using a purified antibody and recombinant antibodies.

Fig. 3B is a photograph showing the results of Western blot analysis using a purified antibody and recombinant antibodies.

Fig. 4 shows photographs showing the results of immunohistostaining of human colon cancer tissues using a purified antibody and recombinant antibodies.

Fig. 5 shows photographs showing the results of immunohistostaining of human normal small intestine tissues using a purified antibody and recombinant antibodies.

Fig. 6 shows photographs showing the results of immunohistostaining of human normal colon tissues using a purified antibody and recombinant antibodies.

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Fig. 7A shows the antitumor effects of recombinant antibodies cA33 and rec263 against a mouse tumor-bearing model wherein COLO205 cells were implanted.

Fig. 7B shows the antitumor effects of recombinant antibodies cA33 and rec263 against a mouse tumor-bearing model wherein NCI-H508 cells were implanted.

Fig. 7C shows the antitumor effects of purified hybridoma antibodies 125M10AA, 125M165DAAA, and 125M96ABA against a mouse tumor-bearing model wherein COLO205 cells were implanted.

Fig. 7D shows the antitumor effects of recombinant antibodies recN26 and recM165 against a mouse tumor-bearing model wherein NCI-H508 cells were implanted.

Fig. 7E shows the antitumor effects of recombinant antibodies recN26 and recM165 against a mouse tumor-bearing model wherein NCI-H508 cells were implanted with Matrigel.*

Fig. 7F shows the antitumor effects of recombinant antibodies recM10 and recQ54 against a mouse tumor-bearing model wherein NCI-H508 cells were implanted with Matrigel.

Preferred Embodiments of the Invention

Hereafter, the present invention is described in detail.

For A33, a mouse anti-A33 antibody and a humanized anti-A33 antibody have already been obtained. Phase I clinical tests conducted on colon cancer patients using the mouse anti-A33 antibody (see Welt S. et al., J. Clinical Oncology (1994), 12, 1561-1571; Welt S. et al., J. Clinical Oncology (1996), 14, 1787-1797) and the humanized A33 antibody (see Welt S. et al., Clinical Cancer Res. (2003), 9, 1338-1346; Welt S. et al., Clinical Cancer Res. (2003), 9, 1347-1353) have been reported. However, HAMA or HAHA was produced at very high probabilities in patients receiving the antibody, and no further clinical tests were conducted following these. However, very interestingly, no HAHA production was observed in the cases of patients confirmed to

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have shown tumor reactivity in the clinical test using the humanized anti-A33 antibody.

The novel human anti-A33 monoclonal antibody of the present invention is a complete human antibody. Hence, the antibody of the present invention avoids in advance the problem that always arises upon the use of a mouse antibody or a humanized antibody regarding antigenicity against the portion comprising the mouse sequence. Specifically, in the above clinical test report, HAHA was produced because of the use of the humanized antibody. However, since the novel human anti-A33 monoclonal antibody of the present invention is a complete human antibody, the antigenicity of an antibody can be avoided, and no HAHA is produced. Thus, significant antitumor effects of the antibody can be expected for colon cancer patients.

Examples of antibody classes used herein include immunoglobulin G(IgG), A(IgA), E(IgE), and M(IgM). A preferable antibody class to be used herein is IgG. Furthermore, as an IgG subclass, IgG1, IgG2, IgG3, or IgG4 is used. Preferably, IgG1, IgG2, or IgG4 is used and further preferably, IgG1 is used.

Hereafter, the present invention will be described in detail through clarification of the meanings of terms and phrases used in the present invention.

1. A33 and anti-A33 antibody

The antibody of the present invention is a class I cell membrane protein and is an antibody against A33, which is a member of the Ig superfamily.

“Antibody binding to A33” in the present invention refers to an antibody that has reactivity to A33 or a part of A33 or an antibody that recognizes A33 or a part of A33. “Functional fragment” in the present invention refers to a portion (partial fragment) of an antibody, which retains 1 or more actions of an antibody on its corresponding antigen. Specific examples of such fragment include F(ab')₂, Fab', Fab, Fv, disulfide-stabilized Fv, single-chain Fv(scFv), and multimers thereof (D. J. King., Applications and Engineering of Monoclonal Antibodies., 1998 T. J. International Ltd). Alternatively, “functional fragment” refers to a fragment of an antibody, which is capable of binding to an antigen.

"Human antibody" in the present invention refers to an antibody that is an expression product of a human-derived antibody gene. As described later, a human antibody can be obtained by introducing a human antibody gene locus and then administering an antigen to a transgenic animal capable of producing a human-derived antibody. An example of such transgenic animal is a mouse. A method for producing such mouse capable of producing a human antibody is described in International Patent Publication WO02/43478, for example.

Examples of the antibody of the present invention include, as described in the following examples, various antibodies that exert antitumor effects against A33-expressing cancer cells, even in low concentrations.

The antibody of the present invention also encompasses a monoclonal antibody comprising a heavy chain and/or light chain having an amino acid sequence derived from the amino acid sequence of a heavy chain or light chain constituting an antibody by deletion, substitution, or addition of 1 or several amino acids. The above-mentioned partial amino acid modification (deletion, substitution, insertion, or addition) can be imparted to the amino acid sequence of the antibody of the present invention by partially modifying the nucleotide sequence encoding the amino acid sequence. Such partial modification of a nucleotide sequence can be imparted by a standard method using known forms of site-specific mutagenesis (Proc Natl Acad Sci U.S.A., 1984 Vol 81: 5662). In addition, an "antibody" in the present invention is an immunoglobulin wherein all regions including the heavy chain variable region and the heavy chain constant region as well as the light chain variable region and the light chain constant region, are derived from a gene encoding an immunoglobulin.

The antibody of the present invention also encompasses an antibody of any immunoglobulin class or isotype.

The anti-A33 antibody of the present invention can be produced by a production method as described below. Specifically, for example, nonhuman mammals such as

human-antibody-producing transgenic mice are immunized with A33, a part of A33 or a conjugate of the part of A33 and an appropriate carrier substance (e.g., bovine serum albumin) for enhancing antigenicity together with, if necessary, an immuno-augmenting agent (e.g., Freund's complete or incomplete adjuvant). As A33, both natural A33 and recombinant A33 can be used. Alternatively, immunization can be performed by introducing a gene encoding A33 and then administering animal cells that overexpress A33 on their cell surfaces. A monoclonal antibody can be obtained by fusing antibody-producing cells obtained from immunized animals to myeloma cells incapable of producing any autoantibody, culturing the thus obtained hybridomas, and then selecting clones that produce the monoclonal antibody showing specific affinity for an antigen used for immunization.

The antibody of the present invention also encompasses an antibody, the class of which is converted to a subclass differing from the original subclass by genetic engineering modification (e.g., see EP314161) known by persons skilled in the art. Specifically, an antibody subclass differing from the original antibody subclass can be obtained by a genetic engineering technique using a DNA encoding a variable region of the antibody of the present invention.

ADCC refers to a type of cytotoxicity induced by activation of macrophages, NK cells, neutrophil cells, or the like that are recognized through the binding of antibody constant regions to Fc receptors expressed on the surfaces of the above cells. In contrast, CDC refers to a type of cytotoxicity induced by activation of a complement system that occurs through binding of an antibody to an antigen. It is known that the intensities of these activities vary depending on antibody subclasses. It is also known that such differences are due to structural differences among antibody constant regions (Charles A. Janeway et al., Immunobiology, 1997, Current Biology Ltd/Garland Publishing Inc.). For example, an antibody exhibiting a low degree of binding to Fc receptor can be obtained by converting the antibody subclass of the present invention to

IgG2 or IgG4. In contrast, an antibody exhibiting a high degree of binding to Fc receptor can be obtained by converting the antibody subclass of the present invention to IgG1 or IgG3. Furthermore, the degree of binding to Fc receptor can be varied by modifying the amino acid sequence of a constant region of the antibody of the present invention by a genetic engineering technique or the binding of a constant region sequence having such amino acid sequence (see Janeway CA. Jr. and Travers P. (1997), Immunobiology, Third Edition, Current Biology Ltd/Garland Publishing Inc). Alternatively, a degree of binding to a complement can also be varied by the same method (see Mi-Hua Tao, et al. 1993. J. Exp. Med). For example, the degree of binding to a complement can be varied by mutating a sequence CCC (that encodes proline (P) at position 331 (based on the EU numbering system (see Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242))) of a heavy chain constant portion to TCC encoding serine (S), so as to substitute proline with serine. In the case of an anticancer agent, for example, it is desired that when an antibody alone is unable to exert activity inducing cell death, such antibody have anti-tumor activity based on antibody-dependent cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) mediated by Fc receptor. It may be more desired when an antibody alone is able to exert activity inducing cell death, such antibody have a low degree of binding to Fc receptor. Furthermore, in the case of an immunosuppressive agent, for example, it is desired that when an antibody three-dimensionally inhibits only the binding of T cells to antigen-presenting cells, such antibody lack ADCC activity or CDC activity. Moreover, when ADCC activity or CDC activity can cause toxicity, an antibody wherein the Fc portion is mutated or the subclass thereof is altered so as to avoid activity that causes toxicity may also be desired.

The present invention encompasses the following steps upon production of monoclonal antibodies: (1) purification of a biopolymer to be used as an immunogen and/or preparation of cells overexpressing an antigen protein on the cell surfaces; (2)

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immunization of animals by injection of an antigen, collection of blood, determination of the antibody titer, determination of timing for extracting the spleen and the like, and then preparation of antibody-producing cells; (3) preparation of myeloma cells; (4) cell fusion of antibody-producing cells and myeloma cells; (5) selection of a hybridoma group producing a target antibody; (6) division into a single cell clone (cloning); (7) if necessary, culture of the hybridoma for producing the monoclonal antibody in a large amount, or breeding of animals wherein the hybridoma is implanted; and (8) examination of the physiological activity and the recognition specificity of the thus produced monoclonal antibody or determination of the properties of the product as a labeling reagent, for example.

Polymorphisms are present in the case of A33. The antibody of the present invention binds to A33 by recognizing all A33 polymorphisms that are currently known. Regardless of differences of A33 polymorphisms among patients, a therapeutic or preventive agent containing the antibody of the present invention can act effectively.

Hereinafter, a method for preparing an anti-A33 monoclonal antibody will be described in detail based on the above steps. However, such method for preparing the antibody is not limited to the following methods. For example, antibody-producing cells and myeloma cells other than splenocytes can also be used.

(1) Purification of antigen

Transformed cell lines are prepared by incorporating a DNA encoding A33 into an expression vector for animal cells and then introducing the expression vector into animal cells. The thus obtained transformed cell line can be directly used as an antigen. Furthermore, since the primary structure of A33 protein is known (GenBank^{*} accession No. NP_005305, SEQ ID NO: 12), a peptide is chemically synthesized from the amino acid sequence of A33 by a method known by persons skilled in the art, following which the product can also be used as an antigen.

Furthermore, cells overexpressing A33 on their cell surfaces can also be

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effective as immunogens, which are prepared by introducing full-length A33 into FM3A cells or L929 cells. pΔEGFP-N1-A33 can be prepared by incorporating a DNA encoding the A33 protein into an expression vector pΔEGFP-N1 for animal cells (where a region encoding an EGFP protein of modified pEGFP-N1 (produced by Becton Dickinson Bioscience Clontech) is deleted). However, such DNA encoding A33, vector, host, and the like are not limited to these examples.

Specifically, a transformed cell line is obtained by transforming FM3A cells or L929 cells with pΔEGFP-N1-A33, followed by culturing. The trait of neomycin resistance acquired by cells into which pΔEGFP-N1 vectors are inserted is confirmed, and the expression of A33 is also confirmed using a mouse anti-human A33 antibody (ATCC No. HB-8779). Hence, FM3A cells or L929 cells overexpressing A33 on the cell surfaces can be prepared using such confirmation results as indices.

(2) Step for preparing antibody-producing cells

The antigen obtained in (1) is mixed with Freund's complete or incomplete adjuvant or an adjuvant such as potassium aluminum sulfate. Experimental animals are immunized with the thus obtained mixture as an immunogen. As experimental animals, transgenic mice capable of producing a human-derived antibody are most suitably used. Such mice are described in document of Tomizuka et al (Tomizuka. et al., Proc Natl Acad Sci U.S.A., 2000 Vol 97: 722).

The route of administration of an immunogen upon immunization of mice may be subcutaneous injection, intraperitoneal injection, intravenous injection, intracutaneous injection, intramuscular injection, footpad injection, or the like. Intraperitoneal injection, footpad injection, or intravenous injection is preferred.

Immunization can be performed once or several times repeatedly at appropriate intervals (preferably at 2-week to 4-week intervals). Subsequently, antibody titers against the antigen in the sera of immunized animals are determined. The effects of the subsequent procedures can be increased with the use of an animal with a sufficiently

elevated antibody titer as a supply source of antibody-producing cells. In general, it is preferable to use antibody-producing cells derived from an animal 3 to 5 days after final immunization for subsequent cell fusion.

Examples of methods for determining antibody titers that may be employed herein include various known techniques such as a radioimmunoassay method (hereinafter referred to as the "RIA method"), an enzyme-linked immunosorbent assay method (hereinafter referred to as the "ELISA method"), a fluorescence antibody method, and a passive haemagglutination method. In view of detection sensitivity, rapidity, accuracy, possible automation of procedures, and the like, the RIA method or the ELISA method is more suitable.

Antibody titer can be determined in the present invention by procedures described below, when the ELISA method is employed, for example. First, an antigen against a human antibody is adsorbed on the solid phase surface of a 96-well plate for ELISA or the like. Furthermore, some areas on the solid phase surface at which the antigen is not adsorbed are covered with a protein independent of the antigen (e.g., bovine serum albumin (BSA)). After the surface is washed, the surface is caused to come into contact with a sample (e.g., mouse serum) serially diluted as a primary antibody. An anti-A33 antibody in a sample is caused to bind to the above antigen. An enzyme-labeled secondary antibody against a human antibody is added to bind to the human antibody. After washing, the substrate of the enzyme is added. Antibody titer is calculated through determination of changes or the like in absorbance due to color development based on substrate degradation.

(3) Step for preparing myeloma cells

As myeloma cells, cells incapable of producing any autoantibody derived from mammals such as a mouse, rat, guinea pig, hamster, rabbit, or human can be used. In general, it is preferable to use established cell lines obtained from mice, such as 8-azaguanine-resistant mouse (derived from BALB/c) myeloma cell lines P3X63Ag8U.1

(P3-U1) (Yelton, D. E. et al. *Current Topics in Microbiology and Immunology*, 81, 1-7 (1978)), P3/NSI/1-Ag4-1 (NS-1) (Kohler, G. et al. *European J. Immunology*, 6, 511-519 (1976)), Sp2/O-Ag14 (SP-2) (Shulman, M. et al., *Nature*, 276, 269-270 (1978)), P3X63Ag8.653 (653) (Kearney, J. F. et al., *J. Immunology*, 123, 1548-1550 (1979)), and P3X63Ag8 (X63) (Horibata, K. and Harris, A. W. *Nature*, 256, 495-497 (1975)). These cell lines are sub-cultured in appropriate medium such as 8-azaguanine medium (prepared by adding 8-azaguanine to RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, and fetal calf serum (hereinafter referred to as "FCS")), Iscove's Modified Dulbecco's Medium (hereinafter, referred to as "IMDM"), or Dulbecco's Modified Eagle Medium (hereinafter, referred to as "DMEM"). 3 to 4 days before cell fusion, the cell lines are sub-cultured in normal medium (e.g., DMEM medium containing 10% FCS). On the day of cell fusion, 2×10^7 or more cells are maintained.

(4) Cell fusion

Antibody-producing cells are plasma cells and the lymphocytes that are the precursor cells thereof. Antibody-producing cells may be obtained from any sites of an individual body and can be obtained generally from the spleen, lymph nodes, bone marrow, tonsil, or peripheral blood, an appropriate combination thereof, or the like. Splenocytes are most generally used.

After final immunization, a site at which antibody-producing cells are present, such as the spleen, is excised from a mouse for which a predetermined antibody titer has been obtained, so that splenocytes, which are antibody-producing cells, are prepared. Next, the splenocytes are fused to myeloma cells. Currently, the most generally conducted means for fusing such splenocytes to myeloma cells obtained in step (3) is a method using polyethylene glycol, which is characterized by relatively low cytotoxicity and simple procedures for cell fusion. This method comprises the following procedures, for example.

Splenocytes and myeloma cells are washed well with serum-free culture medium (e.g., DMEM) or phosphate buffered saline (hereinafter, referred to as PBS), mixed until the ratio of the number of cells of splenocytes to myeloma cells reaches approximately 5:1 to 10:1, and then subjected to centrifugation. The supernatant is removed and then precipitated cell groups are loosened well. 1 mL of serum-free culture medium containing 50% (w/v) polyethylene glycol (with a molecular weight between 1000 and 4000) is added dropwise while agitating the solution. Subsequently, 10 mL of serum-free culture medium is gently added, followed by centrifugation. The supernatant is discarded again. The precipitated cells are suspended in normal medium (hereinafter, referred to as HAT medium) containing an appropriate amount of a hypoxanthine-aminopterin-thymidine (hereinafter, referred to as HAT) solution and human interleukin-6 (hereinafter, referred to as IL-6). The suspension is dispensed to each well of a culture plate (hereinafter, referred to as plate), followed by approximately 2 weeks of culture in the presence of 5% carbonic acid gas at 37°C. During the culture period, HAT medium is appropriately supplemented.

(5) Selection of hybridoma groups

When the above myeloma cells are of a 8-azaguanine-resistant cell line, and more specifically, of a hypoxanthine guanine phosphoribosyltransferase (HGPRT)-deficient cell line, non-fused myeloma cells and the fusion cells of multiple myeloma cells are unable to survive in medium containing HAT. On the other hand, fusion cells of multiple antibody-producing cells or hybridomas of antibody-producing cells and myeloma cells can survive. However, such fusion cells of multiple antibody-producing cells have limited lifetime. Accordingly, through continuation of culture in medium containing HAT, only hybridomas that are the fusion cells of antibody-producing cells and myeloma cells survive. As a result, such hybridomas can be selected.

For hybridomas that grow in the form of colonies, HAT medium is exchanged

with medium from which aminopterin is excluded (hereinafter, referred to as HT medium). Subsequently, a portion of the culture supernatant is collected and then anti-A33 antibody titer is determined by the ELISA method, for example. However, when the above fusion protein is used as an antigen for ELISA, clones that produce an antibody specifically binding to the Fc region of human IgG should be eliminated so as to avoid selection of such clones. The presence or the absence of such clones can be confirmed by ELISA or the like using the Fc region of human IgG as an antigen.

As described above, the method using the 8-azaguanine-resistant cell line is described as an example. Other cell lines can also be used, depending on the method used for selecting a hybridoma, and the composition of medium to be used in such a case will vary.

(6) Cloning step

Hybridomas that are revealed to produce a specific antibody as a result of determination of antibody titers by a method similar to the method described in (2) are transferred to another plate, followed by cloning. Examples of a cloning method to be used herein include a limiting dilution method that involves performing dilution so that each well of a plate then contains one hybridoma, and culturing the hybridomas; a soft agar method that involves culturing in soft agar medium and then harvesting colonies, a method that involves extracting each cell using a micromanipulator and then culturing each cell; and a "sorter clone" method that involves separating cells one-by-one with the use of a cell sorter. The limiting dilution method is convenient and is often used.

For wells for which antibody titers are observed, cloning is repeated 2 to 4 times by a limiting dilution method, for example. Cells for which antibody titers are stably observed are selected as cells of the hybridoma line producing the anti-A33 monoclonal antibody.

In addition, mouse-mouse hybridomas that are the human anti-A33 monoclonal antibody-producing cells of the present invention—125M10AA, 125M165DAAA,

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125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA—were deposited on August 24, 2004, with the International Patent Organism Depositary (IPOD) (Central 6, 1-1, Higashi 1, Tsukuba, Ibaraki, Japan), National Institute of Advanced Industrial Science and Technology (AIST), under accession Nos. FERM BP-10107 (denotation for identification: M10), FERM BP-10106 (denotation for identification: M165), FERM BP-10108 (denotation for identification: M96), FERM BP-10109 (denotation for identification: N26), FERM BP-10104 (denotation for identification: Q47), FERM BP-10105 (denotation for identification: Q54), and FERM BP-10103 (denotation for identification: R5), respectively.

(7) Preparation of monoclonal antibodies by culture of hybridomas

After completion of cloning, HT medium is exchanged with normal medium and then hybridomas are cultured. Large-scale culture is performed by rotation culture using large culture bottle, spinner culture, or culture using a hollow fiber system or the like. The supernatant obtained by such large-scale culture is purified by a method known by persons skilled in the art, such as gel filtration, so that the anti-A33 monoclonal antibody can be obtained. Furthermore, the hybridomas are proliferated intraperitoneally in mice (e.g., BALB/c) of the same line or nu/nu mice, rats, guinea pigs, hamsters, rabbits, or the like, so that ascites containing the anti-A33 monoclonal antibody in large amounts can be obtained. As a convenient method for purification, a commercial monoclonal antibody purification kit (e.g., a MAbTrap^{*} GII kit; produced by Amersham Pharmacia Biotech) or the like can also be used.

The thus obtained monoclonal antibody has high antigen specificity against A33.

(8) Determination of monoclonal antibody

The isotype and subclass of the thus obtained monoclonal antibody can be determined as follows. Examples of an identification method include an Ouchterlony method, an ELISA method, and an RIA method. The Ouchterlony method is convenient, but requires a procedure for condensing the concentration of a monoclonal antibody

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when the concentration is low. In the meantime, when the ELISA method or the RIA method is used, the culture supernatant is directly caused to react with antigen-adsorbed solid phase. The isotype and the subclass of a monoclonal antibody can then be identified using antibodies corresponding to various immunoglobulin isotypes and subclasses as secondary antibodies.

Furthermore, protein determination can be performed by a Folin-Lowry method, which is a method that involves calculation based on absorbance at 280 nm ($1.4 \text{ (OD } 280) = \text{immunoglobulin (1 mg/mL)}$)).

Identification of an epitope that is recognized by a monoclonal antibody can be performed as described below. First, various partial structures of molecules that a monoclonal antibody recognizes are prepared. Examples of a method for preparing such partial structures include: a method by which various partial peptides of a molecule are prepared using a known oligopeptide synthesis technique; and a method by which a DNA sequence encoding a target partial peptide is incorporated into a suitable expression plasmid using a gene recombination technique and then the peptide is produced within or outside a host such as *Escherichia coli*. In general, both methods are used in combination for the above purpose. For example, a series of polypeptides are prepared through sequential shortening of the C-terminus or the N-terminus of an antigen protein by appropriate lengths using a gene recombination technique known by persons skilled in the art. The reactivity of a monoclonal antibody against each of these polypeptides is examined, so that a rough recognition site is determined.

Various oligopeptides corresponding to such sites, variants of such peptides, and the like are further subsequently synthesized using an oligopeptide synthesis technique known by persons skilled in the art. An epitope is determined through examination of the binding of a monoclonal antibody (contained as an active ingredient in a preventive or therapeutic agent of the present invention) to such peptides or through examination of the competitive inhibition activity of a peptide against the binding of such monoclonal

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antibody to an antigen. As a convenient method for obtaining various types of oligopeptides, commercial kits (e.g., SPOTs^{*} kit (produced by GenoSys Biotechnology) and a series of multipin peptide synthesis kits (produced by Chiron Corporation) using a multipin synthesis method) can be used.

Furthermore, a gene encoding a human monoclonal antibody is cloned from antibody-producing cells such as hybridomas, and then the gene is incorporated into an appropriate vector. The vector is then introduced into a host (e.g., mammalian cell lines, *Escherichia coli*, yeast cells, insect cells, and plant cells). Hence, a recombinant antibody produced using a gene recombination technique can also be prepared (P. J. Delves., *Antibody Production Essential Techniques*, 1997 Wiley, P. Shepherd, and C. Dean., *Monoclonal Antibodies*, 2000 Oxford University Press, J. W. Goding., *Monoclonal Antibodies: Principles and Practice*, 1993 Academic Press).

The present invention also encompasses nucleic acids containing the gene sequences of the antibodies of the present invention, which are possessed by hybridomas producing the antibodies. In particular, it also encompasses the nucleic acids of the heavy chain variable regions and the light chain variable regions of the antibodies produced by the hybridomas of the present invention described later. "Nucleic acid(s)" used herein include DNA and RNA.

A method employed for preparation of a gene encoding a monoclonal antibody from a hybridoma involves preparing DNAs encoding monoclonal antibody L chain V region, L chain C region, H chain V region, and H chain C region by the PCR method, or the like. As primers, oligo DNAs designed from the anti-A33 antibody gene or the amino acid sequence thereof can be used. As a template, a DNA prepared from a hybridoma can be used. These DNAs are incorporated into one appropriate vector, and then the vector is introduced into a host for expression. Alternatively, these DNAs are separately incorporated into appropriate vectors, followed by co-expression of the DNAs.

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As a vector, a phage or a plasmid that is autonomously replicable in host microorganisms is used. Examples of a plasmid DNA include plasmids derived from *Escherichia coli*, *Bacillus subtilis*, or yeast, while the phage DNA may be λ phage.

Hosts that are used for transformation are not particularly limited as long as they can express a target gene. Examples of such hosts include bacteria (e.g., *Escherichia coli* and *Bacillus subtilis*), yeast, animal cells (e.g., COS cells and CHO cells), and insect cells.

Methods for introducing genes into hosts are known. Examples of such methods include arbitrary methods such as a method using calcium ions, an electroporation method, a spheroplast method, a lithium acetate method, a calcium phosphate method, and a lipofection method. In addition, examples of a method for introducing genes into animals described later include a microinjection method, a method by which genes are introduced into ES cells using the electroporation or lipofection method, and a nuclear transplantation method.

In the present invention, the anti-A33 antibody can be obtained by culturing a transformant and then harvesting the antibody from the culture product. "Culture product(s)" refers to (a) culture supernatant, (b) cultured cells, cultured microbial bodies or disrupted products thereof, or (c) secretion products of the transformant. To culture a transformant, medium appropriate for a host is used and a static culture method, a roller bottle culture method, or the like is employed.

After culture, when a target antibody protein is produced within microbial bodies or cells, the antibody is harvested by disrupting the microbial bodies or cells. In addition, when a target antibody is produced out of microbial bodies or cells, the culture solution is used intact, or microbial bodies or cells are removed by centrifugation or the like. Thereafter, a target antibody can be isolated and purified from the culture product by a single general biochemical method or an appropriate combination of such biochemical methods using various types of chromatography, which are used for protein

isolation and purification.

Furthermore, a technique for producing transgenic animals is used to produce an animal host wherein the gene of a target antibody is incorporated in an endogenous gene, such as transgenic cattle, transgenic goats, transgenic sheep, or transgenic pigs. A monoclonal antibody derived from the antibody gene can also be obtained in large amounts from milk secreted from such transgenic animals (Wright, G., et al., (1991) Bio/Technology 9, 830-834). When a hybridoma is cultured *in vitro*, the hybridoma is proliferated, maintained, and stored in accordance with various conditions, including the properties of cell types to be cultured, the purposes of experiment and research, culture methods, and the like. Such culture can be performed using known nutrition medium that is used for production of a monoclonal antibody in culture supernatant or all types of nutrition medium induced and prepared from known basic medium.

(9) Antibody Properties

The antibody of the present invention has one of the following properties.

(a) ADCC test

In the presence of normal human peripheral blood mononuclear cells, the antibody of the present invention exerts antibody-dependent cellular cytotoxicity (ADCC) against A33-expressing human cancer cells.

(b) CDC test

In the presence of human serum-derived complements, the antibody of the present invention exerts complement-dependent cytotoxicity (CDC) against A33-expressing human cancer cells.

(c) *In vivo* test

The antibody of the present invention exerts antitumor effects against non-human animals bearing A33-expressing human cancer cells.

(d) Competition test

The antibody of the present invention is (i) strongly competitive (blocker), (ii)

weakly competitive (partial blocker), or (iii) not competitive (non-blocker) with chimeric anti-A33 (comprising the heavy chain variable region and the light chain variable region of an antibody produced by hybridoma ATCC HB-8779 and the heavy chain constant region and the light chain constant region of human IgG1).

(e) Immunohistochemical test

The antibody of the present invention results in the staining of human adult colon cancer tissues, human adult normal colon tissues, and human normal small intestine tissues.

Examples of such antibodies include antibodies produced by hybridomas 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA, for example. 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA were deposited on August 24, 2004 with the International Patent Organism Depositary (IPOD) (Central 6, 1-1, Higashi 1, Tsukuba, Ibaraki, Japan), the National Institute of Advanced Industrial Science and Technology (AIST) under accession Nos. FERM BP-10107 (denotation for identification: M10), FERM BP-10106 (denotation for identification: M165), FERM BP-10108 (denotation for identification: M96), FERM BP-10109 (denotation for identification: N26), FERM BP-10104 (denotation for identification: Q47), FERM BP-10105 (denotation for identification: Q54), and FERM BP-10103 (denotation for identification: R5), respectively.

2. Pharmaceutical Compositions

A pharmaceutical composition containing the human anti-A33 antibody of the present invention is also within the scope of the present invention. Such formulation preferably contains a physiologically acceptable diluent or carrier in addition to the antibody, and may be a mixture thereof with a different antibody or a different drug such as an antibiotic agent. Examples of an appropriate carrier include, but are not limited to, physiological saline, phosphate buffered physiological saline, a phosphate buffered

physiological saline glucose solution, and buffered physiological saline. Alternatively, the antibody may be freeze-dried and, when needed, reconstituted by the addition of an aqueous buffer as described above. Such preventive or therapeutic agent can be administered through various routes for administration. Examples of such routes for administration include oral administration with the use of tablets, capsules, granules, powders, syrups, or the like and parenteral administration with the use of injections, drops, suppositories, or the like.

The dose of such pharmaceutical composition differs depending on symptom, age, body weight, and the like. Generally, in the case of oral administration, a dose ranging from approximately 0.01 mg to 1000 mg per day for an adult and can be administered once or several separate times. In the case of parenteral administration, a dose ranging from approximately 0.01 mg to 1000 mg per administration can be administered via subcutaneous injection, intramuscular injection, or intravenous injection.

The present invention also encompasses the above method for preventing or treating diseases using the antibody or the pharmaceutical composition of the present invention. Furthermore, the present invention also encompasses the use of the antibody of the present invention for production of the above preventive or therapeutic agent for diseases.

Tumors that can be prevented or treated with the use of the antibody or a functional fragment thereof of the present invention are colorectal cancer, colon cancer, rectal cancer, gastric cancer, pancreatic cancer, breast cancer, melanoma, renal cell cancer, cervical cancer, endometrial cancer, ovarian cancer, esophageal cancer, prostatic cancer, testicular cancer, mesothelial cancer, and the like. The number of tumor type to which the antibody of the present invention is applied is not limited to a single type. The antibody can also be applied to a case where tumors of a plurality of types are developed simultaneously.

3. Pharmaceutical preparation examples

The molecule of the present invention can be used as an ampule of a sterile solution or a suspension prepared by dissolving the molecule in water or a pharmacologically acceptable solution other than water. Moreover, such ampule is filled with a sterile powdery pharmaceutical preparation (preferably, the molecule of the present invention is freeze-dried) and then the ampule may be diluted with a pharmacologically acceptable solution when it is used.

The present invention will be described in more detail below with reference to examples. However, the present invention is not limited to embodiments described in the examples.

Example 1 Preparation of mouse anti-A33 antibody

A mouse anti-A33 antibody was prepared for use as a positive control antibody in screening for hybridomas producing human monoclonal antibodies and various experiments. An AS33 hybridoma (American Type Culture Collection (ATCC) No. HB-8779) producing the mouse anti-A33 antibody was purchased from ATCC. The hybridoma was cultured according to instructions included in the ATCC product. The hybridoma was freeze-dried. Subsequently, the AS33 hybridoma was acclimatized in eRDF medium (produced by Kyokuto Pharmaceutical Industrial) containing 10% Low IgG Fetal Bovine Serum (produced by HyClone). The acclimatized hybridoma was cryopreserved. Next, for the purpose of antibody purification, a part of the cryopreserved product was acclimatized in eRDF medium (produced by Kyokuto Pharmaceutical Industrial) containing cattle insulin (5 µg/ml, produced by Gibco BRL), human transferrin (5 µg/ml, produced by Gibco BRL), ethanolamine (0.01 mM, produced by Sigma), sodium selenite (2.5×10^{-5} mM, produced by Sigma), and 1% Low IgG Fetal Bovine Serum (produced by HyClone). After culture in a flask, the culture supernatant was collected. The concentration of the purified antibody derived from the

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hybridoma in the collected supernatant was obtained by measurement of absorbance at 280 nm and calculation with 1.4 OD being equivalent to 1 mg/mL (antibody concentration).

Example 2 Preparation of chimeric anti-A33 antibody

A chimeric anti-A33 antibody having human IgG1 heavy chain and human IgG1 light chain constant regions was prepared as a positive control antibody for use in screening for hybridomas producing human monoclonal antibodies or various experiments.

(1) cDNA cloning of chimeric anti-A33 antibody gene and construction of expression vector

The hybridoma AS33 producing the mouse anti-A33 antibody purchased in Example 1 was cultured in DMEM medium (produced by Gibco BRL) containing 10% Fetal Bovine Serum (produced by HyClone), and then total RNA was purified using an RNA extraction reagent ISOGEN^{*} (produced by NIPPON GENE) according to the relevant protocols. Next, polyA+RNA was purified from the total RNA using OligotexTM-dT30 <Super> (produced by TAKARA BIO). Cloning experiments were conducted using the thus obtained polyA+RNA (2.5 µg) as a material and a SMART RACE cDNA Amplification Kit (Becton Dickinson Bioscience Clontech) according to the instructions included therein. The cDNA of a variable region of the antibody gene was thus obtained.

1) Synthesis of 1st strand cDNA

polyA+RNA	(2.5 µg)/3 µl
5'-CDS primer	1 µl
SMART II A oligo	1 µl

A reaction solution having the above composition was subjected to 2 minutes of incubation at 70°C. The following reagents and enzymes were added, and then

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incubation was performed for 1.5 hours at 42°C, followed by cDNA synthesis.

5X First-Strand buffer	2 µl
DTT (20 mM)	1 µl
dNTP Mix (10 mM)	1 µl
PowerScript [*] Reverse Transcriptase	1 µl

After completion of the reaction, 100 µl of Tricine Buffer was added and then incubation was performed at 72°C for 7 minutes.

2) Amplification of heavy chain and light chain genes by PCR

The thus obtained cDNA was used as a template for PCR. A primer set used for PCR was composed of: the PCR primer (for H chain: GPAHvR3Nhe (5'-GCC CTT GGT GCT AGC TGA AGA GAC GGT GAC CAG AGT CCC TTG-3') (SEQ ID NO: 1) or for L chain: GPALvR3Bsi (5'-GTG CAC GCC GCT GGT CAG GGC GCC TG-3') (SEQ ID NO: 2)) specific to the 3' terminus of the mouse anti-A33 antibody heavy chain (hereinafter, a "heavy chain" may also be referred to as an "H chain") variable region DNA or the 3' terminus of the mouse anti-A33 antibody light chain (hereinafter, a "light chain" may also be referred to as an "L chain") variable region DNA; and a UPM primer (oligonucleotide complementary to the common sequence, which is prepared at the 5' terminus of the synthesized cDNA) included in a SMART RACE cDNA Amplification Kit. The H chain leader sequence and variable region (hereinafter, also referred to as "HV") were amplified by PCR. Further, the L chain leader sequence and variable region (hereinafter, also referred to as "LV") were amplified by PCR. cDNA amplification was performed using KOD-Plus-DNA polymerase (produced by TOYOBO), and the following reaction solution was prepared.

Sterile H ₂ O	29.5 µl
cDNA	2.5 µl
KOD-Plus-buffer (10X)	5 µl
dNTP Mix (2 mM)	4 µl

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MgSO ₄ (25 mM)	2 µl
KOD-Plus-DNA polymerase (1 unit/µl)	1 µl
Universal primer A mix (UPM) (10X)	5 µl
Gene specific primers (GSP)	1 µl
Total volume	50 µl

A thermal cycling amplification reaction was performed under the following conditions.

5 cycles:

94°C for 30 sec

72°C for 1 min

5 cycles:

94°C for 30 sec

70°C for 30 sec

72°C for 1 min

25 cycles:

94°C for 30 sec

68°C for 30 sec

72°C for 1 min

The thus amplified PCR fragments were harvested by ethanol precipitation, harvested by agarose gel electrophoresis, and then purified using a QIAquick^{*} Gel Extraction Kit (produced by QIAGEN), which is a DNA purification kit using a membrane. The thus purified HV and LV amplified fragments were separately subcloned into a PCR 4 Blunt-TOPO^{*} vector of a Zero Blunt^{*} TOPO PCR Cloning Kit (produced by Invitrogen). The nucleotide sequences of the insert DNAs were analyzed for the plasmid DNAs of the thus obtained clones. For determination of the DNA nucleotide sequences, M13FW (5'-GTA AAA CGA CGG CCA GTG-3') (SEQ ID NO: 3) and M13RV (5'-CAG GAA ACA GCT ATG AC-3') (SEQ ID NO: 4) were used as primers. The antibody amino acid sequences encoded by the thus determined HV and

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LV gene regions completely matched the amino acid sequences of the mouse anti-A33 antibody (Br J Cancer. 1995 Dec 72 (6): 1364-72) variable regions reported by King D. J. et al.

(3) Construction of expression vector (N5KG1_mVhCA33) for chimeric anti-A33 antibody

The mouse anti-A33 antibody HV was amplified by PCR (94°C for 3 minutes → 94°C for 10 seconds and 68°C for 45 seconds (35 cycles) → 72°C for 7 minutes) using a plasmid DNA containing the thus obtained antibody HV chain as a template and primers (of a primer set for amplification: GPAHv2F5Sal (5'-AGA GAG AGG TCG ACC CAC CAT GAA CTT TGG GCT GAG CTT AGT T-3') (SEQ ID NO: 5) and GPAHvR3Nhe (SEQ ID NO: 1)) designed to add restriction enzyme sites to the termini for ligation. The amplified HV fragments were purified, and then subcloning was performed into PCR 4 Blunt-TOPO vectors. The DNA nucleotide sequence of the inserted portion was analyzed for the subclones. Thus, plasmid DNAs each having a sequence designed not to differ from the gene sequence used as a template were selected. The plasmid DNAs were digested with restriction enzymes *Sal* I and *Nhe* I. An approximately 440-bp DNA was harvested and purified by agarose gel electrophoresis. In the meantime, an N5KG1-Val Lark vector (a modified vector of IDEC Pharmaceuticals, N5KG1 (US patent 6001358)) was similarly treated with restriction enzymes *Sal* I and *Nhe* I and then subjected to dephosphorylation using alkaline phosphatase (*E. coli* C75) (produced by TAKARA BIO). Subsequently, an approximately 8.9-kb DNA was harvested by agarose gel electrophoresis and a DNA purification kit. These two fragments were subjected to a ligation reaction using a DNA ligation kit Ver 2.1 (produced by TAKARA BIO) and then introduced into *Escherichia coli* DH5α, thereby obtaining transformants. Through screening of the transformants, a clone N5KG1_GPA33Hv (clone #2), into which the target HV had been inserted, was selected. For insertion of LV into the thus obtained N5KG1_GPA33Hv, the plasmid DNA was cleaved sequentially with restriction

enzymes *Bgl* II and *Bsi*W I and then subjected to dephosphorylation. A vector DNA of approximately 9.2 kb was then purified. Meanwhile, the LV region was amplified by PCR using a plasmid DNA containing the mouse anti-A33 antibody LV as a template. GPALv2FBgl (5'-AGA GAG AGA GAT CTC TCA CCA TGG GCA TCA AGA TGG AGT TTC AG-3') (SEQ ID NO: 6) and GPALvR3Bsi (SEQ ID NO: 2) were used as a primer set for amplification. The thus purified and amplified LV fragments were subcloned into PCR 4 Blunt-TOPO. The DNA nucleotide sequence of the inserted portion was analyzed for the subclones. Thus, plasmid DNAs each having a sequence designed not to differ from the gene sequence used as a template were selected. The DNAs were digested with restriction enzymes *Bgl* II and *Bsi*W I. An approximately 400-bp DNA was then harvested and purified by agarose gel electrophoresis. The DNA was ligated to the above N5KG1_A33Hv vector fragment that had been cleaved with restriction enzymes *Bgl* II and *Bsi*W I, and was then introduced into *Escherichia coli*, thereby obtaining transformants. Through screening of the transformants, a clone N5KG1_GPA33HvLv (clone # 2), into which the target LV had been inserted, was selected. The finally obtained chimeric anti-A33 antibody expression plasmid DNA was purified in a large quantity. Thus, it was confirmed that no mutations had occurred in the inserted L chain or H chain DNA fragments, or in the DNA nucleotide sequences of the peripheries of the insertion sites.

DNAs encoding the chimeric anti-A33 heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<Chimeric anti-A33 heavy chain nucleic acid sequence> (SEQ ID NO: 7)

10	20	30	40	50	60
ATGAACTTTG GGCTGAGCTT GATTTTCCTT GTCCTAATTT TAAAAGGTGT CCAGTGTGAA					
70	80	90	100	110	120
GTGAAGCTGG TGGAGTCTGG GGGAGGCTTA GTGAAGCCTG GAGGGTCCCT GAAACTCTCC					

130	140	150	160	170	180
TGTGCAGCCT	CTGGATTTCG	TTTCAGTACC	TATGACATGT	CTTGGGTTCG	CCAGACTCCG
190	200	210	220	230	240
GAGAAGAGGC	TGGAGTGGGT	CGCAACCATT	AGTAGTGGTG	GTAGTTACAC	CTACTATTTA
250	260	270	280	290	300
GACAGTGTGA	AGGGCCGATT	CACCATCTCC	AGAGACAGTG	CCAGGAACAC	CCTATACCTG
310	320	330	340	350	360
CAAATGAGCA	GTCTGAGGTC	TGAGGACACG	GCCTTGTATT	ACTGTGCACC	GACTACGGTA
370	380	390	400	410	420
GTCCCGTTTG	CTTACTGGGG	CCAAGGGACT	CTGGTCACCG	TCTCTTCAGC	TAGC.....

<Chimeric anti-A33 heavy chain amino acid sequence> (SEQ ID NO: 8)

10	20	30	40	50	60
MNFGLSLIFL	VLILKGVQCE	VKLVESGGGL	VKPGGSLKLS	CAASGFAFST	YDMSWVRQTP
70	80	90	100	110	120
EKRLEWVATI	SSGGSYTYYL	DSVKGRFTIS	RDSARNTLYL	QMSSLRSED	ALYYCAPTTV
130	140				
VPFAYWGQGT	LTVSSAS..				

<Chimeric anti-A33 light chain nucleic acid sequence> (SEQ ID NO: 9)

10	20	30	40	50	60
ATGGGCATCA	AGATGGAGTT	TCAGACCCAG	GTCTTTGTAT	TCGTGTTGCT	CTGGTTGTCT
70	80	90	100	110	120
GGTGTGATG	GAGACATTGT	GATGACCCAG	TCTCAAAAAT	TCATGTCCAC	ATCAGTAGGA
130	140	150	160	170	180
GACAGGGTCA	GCATCACCTG	CAAGGCCAGT	CAGAATGTTC	GTACTGTTGT	AGCCTGGTAT
190	200	210	220	230	240
CAACAGAAAC	CAGGGCAGTC	TCCTAAAACA	CTGATTTACT	TGGCCTCCAA	CCGGCACACT
250	260	270	280	290	300

GGAGTCCCTG ATCGCTTCAC AGGCAGTGGG TCTGGGACAG ATTTCACTCT CACCATTAGC
 310 320 330 340 350 360
 AATGTGCAAT CTGAAGACCT GGCAGATTAT TTCTGTCTGC AACATTGGAG TTATCCTCTC
 370 380 390 400
 ACGTTCGGCT CGGGGACAAA GTTGGGAAGTA AAACGT....

<Chimeric anti-A33 light chain amino acid sequence> (SEQ ID NO: 10)

10 20 30 40 50 60
 MGIKMEFQTQ VFVFLWLS GVDGDIVMTQ SQKFMSTSVG DRVSITCKAS QNVRTVVAWY
 70 80 90 100 110 120
 QKPGQSPKT LIYLASNRHT GVPDRFTGSG SGTDFTLTIS NVQSEDLADY FCLQHSYPL
 130 140
 TFGSGTKLEV KR.....

In the heavy chain nucleic acid sequence (SEQ ID NO: 7), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 408 and guanine (G) at position 409. In the heavy chain amino acid sequence (SEQ ID NO: 8), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 136 and alanine (A) at position 137. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 7), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 8), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the chimeric anti-A33 antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 7) ranging from guanine (G) at position 58 to adenine (A) at position 408. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 8) ranging from glutamic acid (E) at position 20 to

serine (S) at position 136.

In the light chain nucleic acid sequence (SEQ ID NO: 9), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 393 and cytosine (C) at position 394. In the light chain amino acid sequence (SEQ ID NO: 10), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 131 and arginine (R) at position 132. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 9), the boundary between the signal sequence and the antibody variable region is located between adenine (A) at position 72 and guanine (G) at position 73. In the light chain amino acid sequence (SEQ ID NO: 10), the boundary between the signal sequence and the antibody variable region is located between guanine (G) at position 24 and aspartic acid (D) at position 25.

Accordingly, the variable region in the chimeric anti-A33 antibody light chain has the nucleic acid sequence (SEQ ID NO: 9) ranging from guanine (G) at position 73 to adenine (A) at position 393. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 10) ranging from aspartic acid (D) at position 25 to lysine (K) at position 131.

The following Table 5 lists the nucleotide sequences of synthetic DNAs.

Host cells were transfected with the thus constructed expression vector for the chimeric anti-A33 recombinant antibody, thereby preparing recombinant-antibody-expressing cells. Host cells to be used for expression were CHO cells of a dhfr-deficient cell line (ATCC CRL-9096), CHO-Ras (Katakura Y., et al., Cytotechnology, 31: 103-109, 1999), or HEK293T (ATCC CRL-11268), for example.

Host cells were transfected with the vector by electroporation, lipofection, or the like. Approximately 2 μ g of the antibody expression vector was linearized using restriction enzymes and then subjected to electroporation using a Bio-Rad electrophoreter under conditions of 350 V and 500 μ F. 4×10^6 CHO cells were thus

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transfected with the gene and then the cells were inoculated on a 96-well culture plate. Lipofection was performed using LipofectAMINE Plus* (produced by Gibco BRL) according to the manual included therein. After transfection with the vector, a drug corresponding to the selection marker used in the expression vector was added and then culture was continued. After confirmation of colonies, an antibody-expressing cell line was selected by the method described in Example 6. Antibody purification was performed from the selected cells as described in Example 8.

Example 3 Preparation of antigen

To obtain cells overexpressing A33 (to be used as an immunogen, for screening for an antibody, or the like) on the cell membranes, a plasmid expression vector for the full-length A33 amino acid sequence was constructed. A DNA encoding A33 was prepared by the PCR method.

a) Construction of full-length A33 expression vector

A plasmid vector pΔEGFP-N1-GPA33 retaining A33-encoding cDNA was constructed to prepare a full-length A33 expression vector. pΔEGFP-N1-GPA33 was constructed by the following method. The complete full-length A33 DNA (GenBank DNA NM_005814: SEQ ID NO: 11 or protein NP_005305: SEQ ID NO: 12) was modified by performing a polymerase chain reaction (PCR) so as to add an *EcoR* I sequence to the 5' terminus and to add a *Not* I sequence and a termination codon to the 3' terminus. 30 cycles of a PCR reaction (94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 60 seconds) were performed using Human Colon Marathon-Ready* cDNA (purchased from Becton Dickinson Bioscience Clontech) as a template, A33-F2 5'-GCAGACGAATTCAAGACCATGGTGGGGAAGAT-3' (SEQ ID NO: 13) and A33-R1 5'-CTCGAGCGGCCGCTCTGCTGCTGGCCTGTCACTGGTCGAGGTG-3' (SEQ ID NO: 14) synthesized as primers, and KOD-plus DNA polymerase (produced by TOYOBO). The thus synthesized sequence was digested with *EcoR* I-*Not* I. The

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resultant was isolated as an *EcoR* I-*Not* I fragment. The fragment was then ligated to pΔEGFP-N1-vector (from which the region encoding EGFP protein of modified pEGFP-N1 (produced by Becton Dickinson Bioscience Clontech) had been deleted) that had been cleaved with the same enzymes. The thus obtained plasmid was named pΔEGFP-N1-GPA33. A 977-bp cDNA was encoded in A33 incorporated in pΔEGFP-N1-GPA33. Hereinafter, GeneAmp® PCR System 9700 (produced by PerkinElmer Japan) was used for regulating the temperatures for all PCR reactions in the examples.

b) Preparation of A33-expressing cells

The two following cell lines were transfected with pΔEGFP-N1-GPA33 constructed in a): FM3A cell line (Japanese Collection of Research Bioresources (JCRB) Cell Bank No. 0701) and the L929 cell line (ATCC No. CCL-1). Thus, 2 types of A33-expressing cells were prepared. The electroporation method was employed for FM3A cells. 5×10^6 FM3A cells were transfected with 20 µg of the pΔEGFP-N1-A33 vector using an electrophoreter (produced by BTX) under conditions of 350 V and 950 µF. The cells were inoculated on a 6-well culture plate. After 48 hours of culture at 37°C under 5.0% carbonic acid gas, G418 (produced by Gibco BRL) was added to the cells at a concentration of 1 mg/mL and then the cells were cultured for 1 week. The A33 antigen expressed on the cell membrane surfaces was confirmed using the culture supernatant of the AS33 hybridoma (ATCC No. HB-8779). Flowcytometer (FCM: produced by Becton, Dickinson and Company) analysis was performed using the culture supernatant of the AS33 hybridoma as a primary antibody and a goat anti-mouse Ig gamma F(ab')₂ antibody (produced by Dako) labeled with R-phycoerythrin as a secondary antibody. Thus, among transfected cells that had acquired the trait of G418 resistance, cells expressing A33 on their cell membrane surfaces were selectively sorted.

L929 cells were transfected using Trans IT-LT1 (produced by TAKARA BIO INC). Transfection was performed by a method described in the related manual.

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After 24 hours of culture at 37°C under 5.0% carbonic acid gas, G418 (produced by Gibco BRL) was added to the cells at a concentration of 1 mg/mL and then culture was performed for 1 week. In a manner similar to that in the case of FM3A cells, the A33 antigen expressed on the cell membrane surfaces was confirmed using the culture supernatant of the AS33 hybridoma. Flowcytometer (FCM: produced by Becton, Dickinson and Company) analysis was performed using the AS33 hybridoma (ATCC No. HB-8779) as a primary antibody, a goat anti-mouse Ig gamma F(ab')₂ antibody (produced by Dako) labeled with R-phycoerythrin as a secondary antibody. Thus, among transfected cells that had acquired the trait of G418 resistance, cells expressing A33 on their cell membrane surfaces were selectively sorted.

Single clones expressing the full-length human A33 antigen at high levels could be obtained from both cell lines. FM3A cells and L929 cells, which had expressed the A33 antigen protein at high levels, were named FM3A/A33 and L929/A33, respectively.

shA33EX-hFc protein was prepared for use as an immunogen or in ELISA upon screening for an antibody.

c) Construction of an expression vector for a soluble A33 human Fc fusion protein outside the cell membrane

To construct an expression vector (hereinafter referred to as shA33EX-hFc) for a soluble A33 human Fc fusion protein outside the cell membrane, a plasmid vector pTracer^{*}-CMV-humanFc-A33EXR retaining cDNA encoding the A33 region outside the cell membrane was constructed. pTracer-CMV-humanFc-A33EXR was constructed by the following method. The DNA of the A33 region outside the cell membrane (SEQ ID NO: 11) containing a secretory signal sequence was modified via a polymerase chain reaction (PCR) so as to add an *EcoR* I sequence to the 5' terminus and to add a *Not* I sequence and a termination codon to the 3' terminus. 30 cycles of a PCR reaction (94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 60 seconds) were performed using pΔEGFP-N1-A33 cDNA prepared in a) as a template, A33-F2 (SEQ ID NO: 13)

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and GPA-EXCRR2 5'-CTCGAGCGGCCGCCAGTTCATGGAGGGAGATCTGACG-3' (SEQ ID NO: 15) synthesized as primers, and KOD-plus DNA polymerase (produced by TOYOBO). The thus synthesized sequence was digested with *EcoR* I-*Not* I. The resultant was isolated as an *EcoR* I-*Not* I fragment. The fragment was then ligated to a pTracer-CMV-humanFc vector (plasmid prepared by introducing FLAG and a human IgG1 Fc region at the *Xba* I and *Apa* I sites of modified pTracer-CMV (produced by Invitrogen Life Technologies)) that had been cleaved with the same enzymes. The thus obtained plasmid was named pTracer-CMV-humanFc-A33EXR.

Oligonucleotides such as PCR primers were all synthesized using a DNA autosynthesizer (model 3948; produced by PerkinElmer Japan, Applied Biosystems Division) according to the manual included therewith (see Matteucci, M. D. and Caruthers, M. H. (1981) J. Am. Chem. Soc. 103, 3185-3191). After completion of synthesis, each oligonucleotide was cleaved from support medium and subjected to deprotection. The thus obtained solution was dried, solidified, and then dissolved in distilled water. This solution was cryopreserved at -20°C until use.

d) Expression and purification of shA33EX-hFc protein

Host cells were transfected with the shA33EX-hFc protein expression vector constructed in c), thereby preparing cells expressing the soluble A33 protein outside the cell membrane. Host cells to be used for expression were CHO cells of a dhfr-deficient cell line (ATCC CRL-9096), CHO-Ras (Katakura Y., et al., Cytotechnology, 31: 103-109, 1999), or HEK293T (ATCC CRL-11268), for example.

Host cells were transfected with the vector by electroporation, lipofection, or the like. Approximately 2 µg of the shA33EX-hFc protein expression vector was linearized using restriction enzymes and then subjected to electroporation using a Bio-Rad electrophoreter under conditions of 350 V and 500 µF. 4×10^6 CHO cells were transfected with the gene, and then the cells were inoculated on a 96-well culture plate. Lipofection was performed using LipofectAMINE Plus (produced by Gibco BRL)

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according to the manual included therewith. After transfection with the vector, a drug corresponding to the selection marker for the expression vector was added and then culture was continued.

The shA33EX-hFc protein was purified from the culture supernatant by the following method. The culture supernatant containing the shA33EX-hFc protein was subjected to affinity purification using Hitrap^{*} Protein A FF (produced by Amersham Pharmacia Biotech), PBS as an adsorption buffer, 20 mM sodium citrate as an elution buffer, and 50 mM sodium chloride (pH 2.7). The eluted fraction was adjusted at pH 5.5 by the addition of a 50 mM sodium phosphate solution (pH 7.0). The thus prepared solution of the soluble A33 protein outside the cell membrane was substituted with PBS using Amicon Ultra-15 (produced by Amicon) and then sterilization by filtration was performed using a membrane filter MILLEX^{*}-GV (produced by Millipore) with a pore size of 0.22 μm . A purified shA33EX-hFc protein was thus obtained. The concentration of the shA33EX-hFc protein was found by measurement of absorbance at 280 nm and calculation with 1.4 OD being equivalent to 1 mg/mL (antibody concentration).

Example 4 Production of human-antibody-producing mice

Mice used for immunization had genetic background such that they were homozygous for both disrupted endogenous Ig heavy chain and κ light chain. Furthermore, the mice simultaneously retained a chromosome 14 fragment (SC20) containing the human Ig heavy chain gene locus and a human Ig κ chain transgene (KCo5). The mice had been produced by crossing mice of line A having the human Ig heavy chain gene locus with mice of line B having the human Ig κ chain transgene. The mice of line A were homozygous for both disrupted endogenous Ig heavy chain and κ light chain and retained the inheritable chromosome 14 fragment (SC20), as described in the report of Tomizuka et al (Tomizuka. et al., Proc Natl Acad Sci U.S.A., 2000 Vol. 97:

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722), for example. Furthermore, the mice of line B were homozygous for both disrupted endogenous Ig heavy chain and κ light chain. Furthermore, the mice of line B retaining the human Ig κ chain transgene (KCo5) were transgenic mice, as described in the report of Fishwild et al (Nat Biotechnol, (1996) 14: 845).

Mice used in the following immunization experiments were obtained by crossing male mice of line A with female mice of line B or by crossing female mice of line A with male mice of line B, in the mouse sera of which the human Ig heavy chain and κ light chain were simultaneously detected (Ishida & Lonberg, IBC's 11th Antibody Engineering, Abstract 2000). In addition, the above human-antibody-producing mice (referred to as KM mice) are available from Kirin Brewery Co., LTD. by contract.

Example 5 Preparation of human monoclonal antibody against A33

Monoclonal antibodies were prepared in this example according to a general method described in "*Tan-Clone-Kotai-Jikken-Manual*" ("Experimental Manual for Monoclonal Antibody") (written by Tamie ANDO et al. and published by Kodansha Scientific, Ltd., Tokyo, Japan (1991)), for example. A33 used as an immunogen was the A33-expressing FM3A cell prepared in Example 1 or the shA33EX-hFc protein. Animals used for immunization were the human-antibody (immunoglobulin)-producing mice produced in Example 2.

For the purpose of preparing the anti-A33 human monoclonal antibody, A33-expressing FM3A cells (1×10^7 cells/mouse) prepared in Example 3 were mixed with an RIBI adjuvant (produced by Corixa Corporation), and then human-antibody-producing mice were initially immunized intraperitoneally with the mixture. Following the initial immunization, the animals were immunized with the same cells and the RIBI adjuvant every week for 8 times in total. 3 days before obtainment of the spleen described later, the shA33EX-hFc protein was administered at 20 μ g/mouse via the caudal vein and recombinant human IL-6 was subcutaneously

administered at 5 µg/mouse.

Furthermore, initial immunization was performed using the mixture of the shA33EX-hFc protein (10 µg/mouse) and a CpG adjuvant (produced by QIAGEN). Following the initial immunization, the same protein and the CpG adjuvant were used for immunization twice every 2 weeks. 2 weeks later, immunization was further performed using only such protein. 3 days before obtainment of the spleen described later, the shA33EX-hFc protein was administered at 10 µg/mouse via the caudal vein.

Furthermore, immunization was performed intraperitoneally with the shA33EX-hFc protein (10 µg/mouse), A33-expressing FM3A cells (5×10^6 cells/mouse), and the RIBI adjuvant, followed by 1 to 4 times of immunization every 2 weeks. 4 days before obtainment of the spleen described later, the shA33EX-hFc protein was administered intraperitoneally at 5 µg/mouse.

The spleen was surgically obtained from each of the thus immunized mice and then added to 10 mL of serum-free DMEM medium (produced by Gibco BRL; hereinafter, referred to as serum-free DMEM medium) containing 350 mg/mL sodium hydrogencarbonate, 50 units/mL penicillin, and 50 µg/mL streptomycin. The resultant was strained through a strainer with a mesh (cell strainer: produced by Falcon) using a spatula. The cell suspension that had passed through the strainer was centrifuged, so as to precipitate the cells. The cells were washed twice with serum-free DMEM medium and then suspended in serum-free DMEM medium, followed by determination of cell count. In the meantime, myeloma cells SP2/0 (ATCC No. CRL-1581) were cultured in DMEM medium (produced by Gibco BRL) containing 10% FCS (produced by Sigma; hereinafter, referred to as serum-containing DMEM medium) at 37°C in the presence of 5% carbonic acid gas, so as not to exceed the cell concentration of 1×10^6 cells/mL. The myeloma cells SP2/0 (ATCC No. CRL-1581) were similarly washed with serum-free DMEM medium and then suspended in serum-free DMEM medium, followed by determination of a cell count. The thus collected cell suspension was mixed with the

mouse myeloma cell suspension at a 5:1 ratio in terms of cell count. After centrifugation, the supernatant was completely removed. 1 mL of 50% (w/v) polyethylene glycol 1500 (produced by Boehringer Mannheim) was slowly added as a fusion agent to the pellets while agitating the solution using the tip of a pipette. 1 mL of serum-free DMEM medium pre-heated at 37°C was slowly added in twice and then 7 mL of serum-free DMEM medium was further added to the resultant. After centrifugation, fusion cells obtained by removal of the supernatant were subjected to screening using a limiting dilution method as described below. Hybridoma selection was performed by culturing the cells in DMEM medium containing 10% FCS, IL-6 (10 ng/mL) (or 10% hybridoma cloning factor (hereinafter, referred to as HCF; produced by BIOBASE)), hypoxanthine (H), aminopterin (A), and thymidine (T) (hereinafter, referred to as HAT; produced by Sigma). Furthermore, single clones were obtained by a limiting dilution method using DMEM medium containing HT (produced by Sigma), 10% FCS, and IL-6 (or 10% HCF). Culture was performed in a 96-well microtiter plate (produced by Becton, Dickinson and Company). Selection (screening) of hybridoma clones producing the anti-A33 human monoclonal antibody and characterization of the human monoclonal antibody produced by each hybridoma were performed through measurement by enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FMC), as described later.

Many hybridomas producing the human monoclonal antibody having the human immunoglobulin γ chain (hIg γ) and the human immunoglobulin light chain κ and having reactivity specific to A33 were obtained as confirmed by Cell ELISA, protein ELISA, and FMC analyses described in Example 6. In addition, each hybridoma clone producing the human anti-A33 monoclonal antibody of the present invention was named using numbers and letters in all the following examples, including this example, and in all tables or figures showing the test results in the examples. Furthermore, numbers and letters with "antibody" attached to one end thereof represent antibodies that are produced

by the relevant hybridomas or recombinant antibodies produced by host cells retaining antibody genes (full-length or a variable region) isolated from the relevant hybridoma. Furthermore, within a contextually clear range, the name of a hybridoma clone may represent the name of an antibody. The following hybridoma clones represent single clones: 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA. 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA were deposited on August 24, 2004, with the International Patent Organism Depositary (IPOD) (Central 6, 1-1, Higashi 1, Tsukuba, Ibaraki, Japan) at the National Institute of Advanced Industrial Science and Technology (AIST) under accession Nos. FERM BP-10107 (denotation for identification: M10), FERM BP-10106 (denotation for identification: M165), FERM BP-10108 (denotation for identification: M96), FERM BP-10109 (denotation for identification: N26), FERM BP-10104 (denotation for identification: Q47), FERM BP-10105 (denotation for identification: Q54), and FERM ABP-10103 (denotation for identification: R5), respectively.

Example 6 Selection of clones producing human anti-A33 monoclonal antibody having the human immunoglobulin γ chain (hIg γ) and the human immunoglobulin light chain κ (Ig κ)

Cell ELISA was performed as follows. FM3A/A33 prepared in Example 3 was added at 1×10^5 per well to a 96-well plate (produced by Falcon). Hybridoma supernatant was added and then incubation was performed at 4°C for 30 minutes. Subsequently, the resultants were washed twice with PBS containing 2% FCS and then a goat anti-human IgG F(ab')₂ antibody labeled with horseradish peroxidase (50 μ g/well: produced by IBL) was added, followed by 30 minutes of incubation at 4°C. The resultants were washed twice with PBS containing 2% FCS and then 100 μ L of a TMB color development substrate (produced by DAKO) was added to each well, followed by

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20 minutes of incubation at room temperature. 0.5 M sulfuric acid (100 μ L/well) was added to each well to stop the reaction. Absorbance at a wavelength of 450 nm (reference wavelength: 570 nm) was measured using a microplate reader (1420 ARVO multilabel counter: produced by WALLAC). Antibody-producing clones showing positive reactions were selected. At this time, FM3A cells expressing no A33 antigen were used as negative controls. Specifically, culture supernatants that had reacted with FM3A/A33 cells but not with FM3A cells were selected as antibody-producing clones that had shown positive reactions.

Furthermore, protein ELISA was performed as follows. 50 μ l of the shA33EX-hFc protein prepared in Example 3 and adjusted at pH 9.4 using a 1 μ g/ml carbonate buffer was added to each well of a 96-well microplate for ELISA (Maxisorp* produced by Nunc). Incubation was performed at room temperature for 1 hour or 4°C overnight, so that the shA33EX-hFc protein was adsorbed to the microplate. Subsequently, the supernatant was discarded, and PBS containing 10% FCS was added to each well, followed by 1 hour of incubation at 37°C. Thus, sites where no shA33EX-hFc protein had bound were blocked. In this manner, the microplate was prepared, wherein each well had been coated with the shA33EX-hFc protein. The culture supernatant (50 μ l) of each hybridoma was added to each well, followed by 1 hour of reaction at room temperature. Each well was washed twice with PBS (PBS-T) containing 0.1% Tween20. Subsequently, a sheep anti-human Igk antibody (50 μ l/well, produced by The Binding Site) labeled with horseradish peroxidase was diluted 2500 fold using PBS (PBS-T) containing 0.1% Tween20.* 50 μ l of the solution was added to each well, followed by 1 hour of incubation at 37°C. The microplate was washed three times with PBS-T. 100 μ l of a TMB color development substrate solution (produced by DAKO) was added to each well, followed by 20 minutes of incubation at room temperature. 0.5 M sulfuric acid (100 μ l/well) was added to each well so as to stop the reaction. Absorbance at a wavelength of 450 nm (reference wavelength: 570 nm) was

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measured using a microplate reader (VersaMax* produced by Molecular Devices Corporation). Antibody-producing clones showing positive reactions were selected.

Furthermore, FMC was performed as follows. The reactivity of hybridoma culture supernatants against human colorectal cancer cell line COLO205 cells expressing the A33 antigen was examined. The COLO205 cell line was suspended at a concentration of 2×10^6 /ml in a PBS staining buffer (SB) containing 0.1% NaN₃ and 2% FCS. The cell suspension (50 µl/well) was dispensed in a 96-well round bottom plate (produced by Becton, Dickinson and Company). The culture supernatant (50 µl) of each hybridoma was added, followed by 30 minutes of incubation on ice. A negative control was prepared depending on each subclass. Specifically, a human IgG1 antibody (produced by Sigma) was adjusted at a concentration of 2 µg/ml with hybridoma culture medium and then 50 µl of the solution was added, followed by 30 minutes of incubation on ice. The resultant was washed twice with SB and then 50 µl of RPE fluorescence-labeled goat anti-human IgG F(ab')₂ antibody (produced by Southern Biotech) was added, followed by 30 minutes of incubation on ice. The resultant was washed once with SB and it was then suspended in 300 µl of a FACS buffer. The mean fluorescence intensity of each cell line was measured by FACS (FACS caliber produced by Becton, Dickinson and Company). As a result, the presence of antibodies binding to A33 expressed on cells was demonstrated because of their strong activity of binding to the cells of the COLO205 cell line.

Example 7 Identification of each monoclonal antibody subclass in the culture supernatant

50 µl of the shA33EX-hFc protein prepared using a 1 µg/ml carbonate buffer (hereinafter, referred to as PBS) was added to each well of a 96-well microplate for ELISA (Maxisorp produced by Nunc). Incubation was performed at room temperature for 1 hour or 4°C overnight, so that the shA33EX-hFc protein was adsorbed to the

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microplate. Subsequently, the supernatant was discarded, and PBS containing 10% FCS was added to each well, followed by 1 hour of incubation at room temperature or at 4°C overnight. Thus, sites where no shA33EX-hFc protein had bound were blocked. In this manner, the microplate was prepared, with each well having been coated with the shA33EX-hFc protein. Subsequently, the microplate was washed twice with PBS (PBS-T) containing 0.1% Tween20. A sheep anti-human IgG1 antibody labeled with horseradish peroxidase, a sheep anti-human IgG2 antibody labeled with horseradish peroxidase, a sheep anti-human IgG3 antibody labeled with horseradish peroxidase, or a sheep anti-human IgG4 antibody labeled with horseradish peroxidase (diluted 1600-, 6400-, 25000-, and 25000-fold, respectively and produced by The Binding Site) was added at 50 µL/well. Incubation was performed for 1.5 hours at room temperature. The microplate was washed three times with PBS-T containing 0.1% Tween20. A substrate buffer (TMB produced by DAKO) was added at 100 µL/well, followed by 20 minutes of incubation at room temperature. Subsequently, 0.5 M sulfuric acid (100 µl/well) was added to stop the reaction. Absorbance at a wavelength of 450 nm (reference wavelength: 570 nm) was measured using a microplate reader (VersaMax produced by Molecular Devices Corporation). The subclass of each clone was determined. Only human anti-A33 antibodies of subclass IgG1 were selected because a high priority was placed on ADCC and CDC.

Table 1 shows the reactivity of only the finally selected clones.

Table 1

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Antibody name	Subclass	Reactivity to COLO205
Anti-DNP-IgG1	IgG1	-
cA33	IgG1	+
263A17	IgG1	+
125M10AA	IgG1	+
125M165DAAA	IgG1	+
125M96ABA	IgG1	+
125N26F6AA	IgG1	+
125Q47BA	IgG1	+
125Q54AAAA	IgG1	+
125R5AAAA	IgG1	+

Example 8 Preparation of each type of antibody

The human anti-A33 monoclonal antibodies obtained from the culture supernatants of hybridomas as described in Example 6 were purified by the following methods. A culture supernatant containing each type of human anti-A33 monoclonal antibody was cultured in SFM medium (produced by Invitrogen) containing 10 % ultra low IgG FBS (produced by Invitrogen). The culture supernatant was subjected to affinity purification using Protein A Fast Flow^{*} gel (produced by Amersham Pharmacia Biotech), PBS as an adsorption buffer, and a 0.02 M glycine buffer (pH 3.6) as an elution buffer. The eluted fraction was adjusted at around pH 7.2 by the addition of 1M Tris (pH 8.0). The thus prepared antibody solution was substituted with PBS using Sephadex^{*} G25 desalting column (NAP column; produced by Amersham Pharmacia Biotech) and then sterilized by filtration using a membrane filter MILLEX-GV (produced by Millipore) with a pore size of 0.22 μ m. Thus, purified human anti-A33 monoclonal antibodies were obtained. The concentration of each purified antibody was obtained by measurement of absorbance at 280 nm and calculation with 1.4 OD being

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equivalent to 1 mg/mL (antibody concentration).

Example 9 Test of the reactivity of each type of purified monoclonal antibody to A33-expressing cells

The reactivity of each type of purified monoclonal antibody obtained in Example 8 to A33-antigen-expressing human colorectal cancer cell line COLO205 cells, LoVo cells (ATCC No. CCL-229), LS174T cells (ATCC No. CL-188), and NCI-H508 cells (ATCC No. CCL-253) was examined by FCM. Human colorectal cancer cell line HT-29 cells (ATCC No. HTB-38) expressing no A33 antigen were also examined as negative control cells. Each cell line was suspended at a concentration of 2×10^6 /ml in a PBS Staining Buffer (SB) containing 0.1% NaN₃ and 2% FCS. The cell suspension (50 µl/well) was dispensed in a 96-well round bottom plate (produced by Becton, Dickinson and Company). 50 µl of each type of purified monoclonal antibody (adjusted using SB at a concentration of 2000, 400, 80, or 16 ng/ml) was added, and then incubation was performed on ice for 30 minutes. A negative control was prepared depending on each subclass. Specifically, a human IgG1 antibody (produced by Sigma) was adjusted using SB at a concentration of 2000, 400, 80, or 16 ng/ml. 50 µl of the solution was added, followed by 30 minutes of incubation on ice. The resultant was washed twice with SB and then 50 µl of an FITC fluorescence-labeled goat anti-human IgG F(ab')₂ antibody (produced by Southern Biotech), followed by 30 minutes of incubation on ice. The resultant was washed once with SB and then the resultant was suspended in 300 µl of a FACS buffer. The mean fluorescence intensity of each cell line was measured by FACS (FACScan produced by Becton, Dickinson and Company).

Table 2 shows the results. For COLO205 cells, the half value of the mean fluorescence intensity was determined to be 90. For LoVo cells, the half value of the mean fluorescence intensity was determined to be 25. For LS174T cells, the half value of the mean fluorescence intensity was determined to be 125. For NCI-H508 cells, the

half value of the mean fluorescence intensity was determined to be 125. When an antibody concentration required to reach such value was $10 \leq x < 100$ ng/ml, the reactivity was represented by +++, when the same was $100 \leq x < 1000$ ng/ml, the reactivity was represented by ++, and when the same was $1000 \leq x < 10000$ ng/ml, the reactivity was represented by +. When no binding was observed, the reactivity was represented by -. Each type of purified monoclonal antibody showed binding to all cells expressing the A33 antigen.

Table 2

Antibody name	COLO205	LoVo	LS174T	NCI-H508	HT-29
Anti-DNP-IgG1	-	-	-	-	-
cA33	+++	+++	++	++	-
263A17	++	++	++	++	-
125M10AA	+++	+++	+++	+++	-
125M165DAAA	+++	+++	++	++	-
125M96ABA	+++	+++	++	++	-
125N26F6AA	+++	+++	++	++	-
125Q47BA	+++	+++	++	++	-
125Q54AAAA	+++	+++	++	++	-
125R5AAAA	+++	+++	++	++	-

Half value of mean fluorescence intensity 90	Half value of mean fluorescence intensity 25	Half value of mean fluorescence intensity 125	Half value of mean fluorescence intensity 125
10 <= x < 100 ng/ml; +++	10 <= x < 100 ng/ml; +++	10 <= x < 100 ng/ml; +++	10 <= x < 100 ng/ml; +++
100 <= x < 1000 ng/ml; ++	100 <= x < 1000 ng/ml; ++	100 <= x < 1000 ng/ml; ++	100 <= x < 1000 ng/ml; ++
1000 <= x < 10000 ng/ml; +	1000 <= x < 10000 ng/ml; +	1000 <= x < 10000 ng/ml; +	1000 <= x < 10000 ng/ml; +
No binding; -	No binding; -	No binding; -	No binding; -

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Example 10 Competition test regarding each type of purified monoclonal antibody and the mouse anti-A33 antibody

Whether or not each type of purified monoclonal antibody obtained in Example 8 recognizes an epitope similar to that of the mouse anti-A33 antibody was examined by a competition test using FCM. The COLO205 cell line was suspended at a concentration of 2×10^6 /ml in a PBS Staining Buffer (SB) containing 0.1% NaN₃ and 2% FCS. The cell suspension (50 µl/well) was dispensed in a 96-well round bottom plate (produced by Becton, Dickinson and Company). The purified mouse anti-A33 antibody prepared in Example 1 was not added or added at a concentration of 100 µg/ml to the wells containing each type of purified monoclonal antibody (1 µg/ml and 50 µl), followed by 30 minutes of incubation on ice. A mouse negative control (containing no purified anti-A33 antibody added thereto) was prepared as follows. A human IgG1 antibody (produced by Sigma) was adjusted at a concentration of 1 µg/ml with SB and then 50 µl of the solution was added, followed by 30 minutes of incubation on ice. The resultant was washed twice with SB and then 50 µl of FITC fluorescence-labeled goat anti-human IgG F(ab')₂ antibody (produced by IBL) was added, followed by 30 minutes of incubation on ice. The resultant was washed once with SB and then the resultant was suspended in 300 µl of a FACS buffer. The mean fluorescence intensity of each cell line was measured by FACS (FACScan* produced by Becton, Dickinson and Company). The inhibition (%) between each type of purified monoclonal antibody and the mouse A33 antibody was calculated by the following formula:

$$\text{Inhibition (\%)} = \{100 - (100 \times \text{mean fluorescence intensity after preincubation with mouse A33 antibody}) / (\text{mean fluorescence intensity after preincubation without mouse A33 antibody})\}.$$

A purified monoclonal antibody showing inhibition (%) of 25% or less was classified as a "non-blocker," one showing inhibition (%) of 25% or more and less than 90% was classified as a "partial blocker," and one showing inhibition (%) of 90% or more was classified as a "blocker." As a result, 263A17, 125M10AA, and 125M96ABA were classified as "non-blockers," 125M165DAAA and 125N26F6AA were classified as

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“blockers,” and 125Q47BA, 125Q54AAAA, and 125R5AAAA were classified as “partial blockers.” Table 3 shows the results.

Table 3

Antibody name	Inhibition (%)	Classification
cA33	94.2	Blocker
263A17	3.7	Non-blocker
125M10AA	5.0	Non-blocker
125M165DAAA	96.0	Blocker
125M96ABA	22.8	Non-blocker
125N26F6AA	98.0	Blocker
125Q47BA	64.1	Partial blocker
125Q54AAAA	46.5	Partial blocker
125R5AAAA	63.9	Partial blocker

Example 11 Method for obtaining normal human mononuclear leukocytes

First, normal human peripheral blood mononuclear cells were prepared using Ficoll* (Ficoll-PaquePLUS: produced by Amersham Pharmacia Biotech) according to a standard method. Normal human blood was collected in a blood collection bag (produced by TERUMO) containing a sodium citrate solution as an anticoagulant. The normal human blood was multi-layered in Ficoll and then specific gravity centrifugation (800G, room temperature, and 15 minutes) was performed to separate monocytes. The intermediate layer was extracted in the form of mononuclear leukocytes, and the extracted mononuclear leukocytes were then diluted with PBS. The diluted solution was repeatedly centrifuged at 200 G for 10 minutes three times, thereby removing blood platelets remaining in the supernatant. Normal human peripheral blood mononuclear cells (hereinafter referred to as PBMC) were obtained by the above method and then used as PBMC for Example 12. Furthermore, CD4+ T cells were isolated from PBMC using a CD4+ T cell Isolation kit II (produced by Miltenyi Biotec) according to the instructions included therein. The remaining cell group was also used as PBMC for

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Example 12.

Example 12 Test of the cytotoxicity of each type of purified monoclonal antibody

Antibody-mediated cytotoxicity was determined as follows. Cytotoxicity (antibody-dependent cellular cytotoxicity, hereinafter referred to as ADCC) against target cells was determined in the presence of cells having killer activity, such as NK cells or neutrophils, and antibodies. Furthermore, cytotoxicity (complement-dependent cytotoxicity, hereinafter referred to as CDC) against target cells was determined in the presence of complements and antibodies. The antibodies used herein were: each type of purified monoclonal antibody prepared in Example 8 and a cA33 recombinant antibody as a control anti-A33 antibody. Furthermore, an anti-DNP IgG1 antibody was used as a negative control.

The method is simply described as follows. Target cells were caused to incorporate radioactive chromium (^{51}Cr) into the cytoplasm and γ -dose was measured to find the amounts of ^{51}Cr that are released in culture solutions because of cell death.

Specifically, 10^6 cells of colorectal cancer cell line COLO205 (ATCC No. CCL-86) and 10^6 NCI-H508 cells (ATCC No. CCL-253) were separately suspended as target cells in 15 μL of fetal calf serum (FCS). 50 μL (37 MBq/mL) of ^{51}Cr -labeled sodium chromate (produced by PerkinElmer; hereinafter referred to as ^{51}Cr) was added, followed by 1 hour of culture at 37°C. Next, 10 mL of medium was added. Discarding of medium by centrifugation was repeated 3 times, thereby eliminating ^{51}Cr that had not been incorporated within the cells.

In the ADCC assay, 5,000 ^{51}Cr -labeled target cells and 500,000 healthy human peripheral blood mononuclear leukocytes obtained by the method described in Example 11 were cultured in a V-bottom 96-well plate (produced by Coaster) at a total volume of 200 μL with antibody having each concentration at 37°C in the presence of 5% CO_2 for 4 hours.

In the CDC assay, 5000 ^{51}Cr -labeled target cells and human serum-derived complements (produced by Sigma) at a final concentration of 5% were cultured in a

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V-bottom 96-well plate at a total volume of 200 μ L with antibody having each concentration at 37°C in the presence of 5% CO₂ for 4 hours.

In both ADCC and CDC assays, the plate was subjected to centrifugation after culture to cause the cells to precipitate. Each type of purified monoclonal antibody was prepared at a concentration of 0.4-500 ng/ml and then 50 μ L of the solution was transferred to a 96-well plate (LumaplateTM-96; produced by Packard Instrument) including a powder scintillator. The resultant was dried at 55°C for 1.5 hours. After confirming that the plate had dried, the plate was covered with a special cover (TopSealTM-A; 96-well microplates; produced by Packard Instrument). The γ -ray dose was measured with a scintillation counter (TopCount^{*}; produced by Packard Instrument).

Fig.1A to Fig. 1D and Table 4 show the results. In the case of ADCC against COLO205 cells, the half value of the specific lysis (%) was determined to be 15%. When an antibody concentration required to reach such value was $1 \leq x < 10$ ng/ml, the cytotoxicity was represented by +++, when the same was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by ++, when the same was $100 \leq x < 1000$ ng/ml, the cytotoxicity was represented by +, and when no specific lysis (%) was obtained, the cytotoxicity was represented by -. In the case of ADCC against NCI-H508 cells, the half value of the specific lysis (%) was determined to be 15%. When an antibody concentration required to reach such value was $1 \leq x < 10$ ng/ml, the cytotoxicity was represented by +++, when the same was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by ++, when the same was $100 \leq x < 1000$ ng/ml, the cytotoxicity was represented by +, and when no specific lysis (%) was observed, the cytotoxicity was represented by -.

In the case of CDC against COLO205 cells, the half value of the specific lysis (%) was determined to be 10%. When an antibody concentration required to reach such value was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by +++, when the same was $100 \leq x < 1000$ ng/ml, the activity was represented by ++, when the same required to reach such value was $x \geq 1000$ ng/ml, the activity was represented by +, and when no specific lysis (%) was obtained, the activity was represented by -. Furthermore, in

the case of CDC against NCI-H508 cells, the half value of the specific lysis (%) was determined to be 25%. When an antibody concentration required to reach such value was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by +++, when the same was $100 \leq x < 1000$ ng/ml, the cytotoxicity was represented by ++, when the same was $x \geq 1000$ ng/ml, the cytotoxicity was represented by +, and when no specific lysis (%) was obtained, the cytotoxicity was represented by -.

In the case of ADCC, cA33 and 125Q54AAAA showed high cytotoxicity, while in the case of CDC, 125M10AA showed high cytotoxicity.

Table 4

Cell name	COLO205			NCI-H508		
Antibody name	ADCC		CDC	ADCC		CDC
Anti-DNP-IgG1	-		-	-		-
cA33	+++		-	+++		-
263A17	++		++	+++		++
125M10AA	++		+++	+++		+++
125M165DAAA	++		+	+++		++
125M96ABA	+		++	++		+++
125N26F6AA	++		-	++		+
125Q47BA	++		++	++		++
125Q54AAAA	+++		++	+++		++
125R5AAAA	++		++	+++		++

Half value of specific lysis (%) 15%	Half value of specific lysis (%) 10%	Half value of specific lysis (%) 15%	Half value of specific lysis (%) 25%
1<=x<10 ng/ml; +++	10<=x<100 ng/ml; +++	1<=x<10 ng/ml; +++	10<=x<100 ng/ml; +++
10<=x<100 ng/ml; ++	100<=x<1000 ng/ml; ++	10<=x<100 ng/ml; ++	100<=x<1000 ng/ml; ++
100<=x<1000 ng/ml; +	x>=1000 ng/ml; +	100<=x<1000 ng/ml; +	x>=1000 ng/ml; +
No specific lysis; -	No specific lysis; -	No specific lysis; -	No specific lysis; -

Example 13 Preparation of the gene encoding each type of monoclonal antibody

(1) cDNA synthesis for each type of monoclonal antibody

Hybridomas 263A17, 125M10AA, 125M165DAAA, 125M96ABA, 125N26F6AA, 125Q47BA, 125Q54AAAA, and 125R5AAAA were separately cultured in DMEM medium (produced by Gibco BRL) containing 10 ng/mL IL-6 or 10% HCF (produced by BIOBASE) and 10% Fetal Bovine Serum (produced by HyClone). After the cells were harvested by centrifugation, ISOGEN (produced by NIPPON GENE) was added and then total RNAs were extracted according to the relevant instruction manual. Variable regions of antibody cDNAs were cloned using a SMART RACE cDNA amplification kit (produced by Becton Dickinson Bioscience Clontech) according to instructions included therein.

1st strand cDNA was prepared using 5 µg of total RNA as a template.

1st strand cDNA synthesis

Total RNA 5 µg/3 µl

5'CDS 1 µl

SMART oligo 1 µl

A reaction solution with the above composition was incubated at 70°C for 2 minutes, and then the following components were added, followed by 1.5 hours of incubation at 42°C.

5 × Buffer 2 µl

DTT 1 µl

DNTP mix 1 µl

PowerScript Reverse Transcriptase 1 µl

Furthermore, 100 µl of a tricine buffer was added and then incubation was performed at 72°C for 7 minutes, thereby obtaining 1st strand cDNA.

(2) PCR amplification of heavy chain and light chain genes and confirmation of the nucleotide sequences

(2)-1: PCR amplification of the heavy chain and light chain genes of hybridoma 263A17 cDNA amplification was performed by preparing the following reaction solution using KOD-Plus-DNA polymerase (produced by TOYOBO).

Sterile H ₂ O	29.5 µl
cDNA	2.5 µl
KOD-Plus- buffer (10X)	5 µl
dNTP Mix (2mM)	4 µl
MgSO ₄ (25 mM)	2 µl
KOD-Plus- (1 unit/µl)	1 µl
Universal primer A mix (UPM) (10X)	5 µl
Gene specific primers (GSP)	1 µl
Total volume	50 µl

The reaction solution with the above composition was adjusted to a final volume of 50 µl with re-distilled water and then subjected to PCR.

The 263A17 heavy chain gene was amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using the UPM primer included in a SMART RACE cDNA Amplification Kit and a IgG1p primer (5'-TCTTGTCCACCTTGGTGTTGCTGGGCTTGTG-3') (SEQ ID NO: 16). Meanwhile, the 263A17 light chain gene was amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using the UPM primer and an hk-2 (5'-GTT GAA GCT CTT TGT GAC GGG CGA GC-3') (SEQ ID NO: 17) primer.

(2)-2: PCR Amplification of heavy chain and light chain genes of hybridomas 125M10AA, 125M96ABA, 125Q47BA, 125Q54AAAA, and 125R5AAAA

Reaction conditions employed herein were similar to those in (2)-1. The 125M10AA, 125M96ABA, 125Q47BA, 125Q54AAAA, and 125R5AAAA heavy chain genes were amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using the UPM primer and the IgG1p primer (SEQ ID NO: 16). Furthermore, a reaction cycle (98°C for 1 second and 68°C for 30 seconds) was repeated 20 times using 1 µl of the reaction solution as a template, a NUPM primer (SMART RACE cDNA amplification Kit; produced by Becton Dickinson Bioscience Clontech), and a IgG2p/G134 primer (5'-TGC ACG CCG CTG GTC AGG GCG CCT GAG TTC C-3') (SEQ ID NO: 18). Meanwhile, the 125M10AA, 125M96ABA, 125Q47BA,

125Q54AAAA, and 125R5AAAA light chain genes were amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using the UPM primer and the hk-2 primer (SEQ ID NO: 17). Furthermore, a reaction cycle (98°C for 1 second and 68°C for 30 seconds) was repeated 20 times using 1 µl of the reaction solution as a template, the NUPM primer, and an hk-5 primer (5'-AGG CAC ACA ACA GAG GCA GTT CCA GAT TTC-3') (SEQ ID NO: 19).

(2)-3: PCR amplification of the heavy chain and light chain genes of hybridomas 125M165DAAA and 125N26F6AA

Reaction conditions employed herein were similar to those in (2)-1. 125M165DAAA and 125N26F6AA heavy chain genes were amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using a UPM primer and an hh-2 primer (5'-GCT GGA GGG CAC GGT CAC CAC GCT G-3') (SEQ ID NO: 20). Furthermore, a reaction cycle (98°C for 1 second and 68°C for 30 seconds) was repeated 20 times using 1 µl of the reaction solution as a template, a NUPM primer, and an hh-4 primer (5'-GGT GCC AGG GGG AAG ACC GAT GG-3') (SEQ ID NO: 21). Meanwhile, the 125M165DAAA and 125N26F6AA light chain genes were amplified by repeating a reaction cycle (98°C for 1 second and 68°C for 30 seconds) 30 times using the UPM primer and the hk-2 primer (SEQ ID NO: 17). Furthermore, a reaction cycle (98°C for 1 second and 68°C for 30 seconds) was repeated 20 times using 1 µl of the reaction solution as a template, an NUPM primer, and the hk-5 primer (SEQ ID NO: 19).

PCR fragments of the heavy and light chains amplified as described above were separately harvested by ethanol precipitation, harvested by agarose gel electrophoresis, and then purified using a QIAquick Gel Extraction Kit (produced by QIAGEN), which is a DNA purification kit that uses membranes. The thus purified HV and LV amplified fragments were separately subcloned into a PCR 4 Blunt-TOPO vector of a Zero Blunt TOPO PCR Cloning Kit (produced by Invitrogen). For the plasmid DNAs of the thus obtained clones, the nucleotide sequences of the insert DNAs were analyzed. M13FW (SEQ ID NO: 3) and M13RV (SEQ ID NO: 4) were used as primers for determination of

the DNA nucleotide sequences.

DNAs encoding the 263A17 heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<263A17 heavy chain nucleic acid sequence> (SEQ ID NO: 22)

10	20	30	40	50	60
ATGGAGTTTG	GGCTGAGCTG	GCTTTTTCTT	GTGGCTATTT	TAAAAGGTGT	CCAGTGTGAG
70	80	90	100	110	120
GTGCAGTTGT	TGGAGTCTGG	GGGAGGCTTG	GTACAGCCTG	GGGGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCCT	CTGGATTCAC	CTTTAGCAGC	TATGCCATGA	GCTGGATCCG	CCAGGCTCCA
190	200	210	220	230	240
GGGAAGGGGC	TGGAGTGGGT	CTCAGCTATT	AGTGCTAGTG	GTGGTAGCAC	ATACTACGCA
250	260	270	280	290	300
GACTCCGTGA	AGGGCCGGTT	CACCATCTCC	AGAGACAATT	CCAAGAACAC	GCTGTATCTG
310	320	330	340	350	360
CAAATGAACA	GCCTGAGAGC	CGAGGACACG	GCCGTATATT	ACTGTGCGAA	AGATCGGATA
370	380	390	400	410	420
GTGGGAGCTA	CGAACTACTA	CTACGGTATG	GACGTCTGGG	GCCAAGGGAC	CACGGTCACC
430	440	450	460	470	480
GTCTCCTCAG	CTAGC. . . .				

<263A17 heavy chain amino acid sequence> (SEQ ID NO: 23)

10	20	30	40	50	60
MEFGLSWLFL	VAILKGVQCE	VQLLES GGGL	VQPGGSLRLS	CAASGFTFSS	YAMSWIRQAP
70	80	90	100	110	120
GKGLEWVSAI	SASGGSTYYA	DSVKGRFTIS	RDNSKNTLYL	QMNSLRAEDT	AVYYCAKDRI
130	140	150	160	170	180
VGATNYYYGM	DVWGQGTTVT	VSSAS. . . .			

<263A17 light chain nucleic acid sequence> (SEQ ID NO: 24)

10	20	30	40	50	60
ATGGACATGA	GGGTCCCCGC	TCAGCTCCTG	GGGCTCCTGC	TGCTCTGGTT	CCCAGGTTCC
70	80	90	100	110	120
AGATGCGACA	TCCAGATGAC	CCAGTCTCCA	CCTTCCGTGT	CTGCATCTGT	AGGAGACAGA
130	140	150	160	170	180
GTCACCATCA	CTTGTCGGGC	GAGTCAGGGT	ATTAGCAGCT	GGTTAGCCTG	GTATCAGCAT
190	200	210	220	230	240
AAACCAGGGA	AAGCCCCAAA	GCTCCTGATC	TATGGTGCAT	CCAGTTTGCA	AAGTGGGGTC
250	260	270	280	290	300
CCATCAAGGT	TCAGCGGCAG	TGGATCTGGG	ACAGATTTCA	CTCTCACCAT	CAGCAGCCTG
310	320	330	340	350	360
CAGCCTGAAG	ATTTTGCAAC	TTACTATTGT	CAACAGGCTA	ATAGTTTCCC	TATCACCTTC
370	380	390			
GGCCAAGGGA	CACGACTGGA	GATTAAACGT			

<263A17 light chain amino acid sequence> (SEQ ID NO: 25)

10	20	30	40	50	60
MDMRVPAQLL	GLLLWFPGS	RCDIQMTQSP	PSVSASVGDR	VTITCRASQG	ISSWLAWYQH
70	80	90	100	110	120
KPGKAPKLLI	YGASSLQSGV	PSRFSGSGSG	TDFTLTISL	QPEDFATYYC	QQANSFPITF
130					
GQGTRLEIKR					

In the heavy chain nucleic acid sequence (SEQ ID NO: 22), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 429 and guanine (G) at position 430. In the heavy chain amino acid sequence (SEQ ID NO: 23), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 143 and alanine (A) at position 144. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 22), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position

58. In the heavy chain amino acid sequence (SEQ ID NO: 23), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the 263A17 antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 22) ranging from guanine (G) at position 58 to adenine (A) at position 429. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 23) ranging from glutamic acid (E) at position 20 to serine (S) at position 143.

In the light chain nucleic acid sequence (SEQ ID NO: 24), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 387 and cytosine (C) at position 388. In the light chain amino acid sequence (SEQ ID NO: 25), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 129 and arginine (R) at position 130. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 24), the boundary between the signal sequence and the antibody variable region is located between cytosine (C) at position 66 and guanine (G) at position 67. In the light chain amino acid sequence (SEQ ID NO: 25), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 22 and aspartic acid (D) at position 23.

Accordingly, the variable region in the 263A17 antibody light chain has the nucleic acid sequence (SEQ ID NO: 24) ranging from guanine (G) at position 67 to adenine (A) at position 387. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 25) ranging from aspartic acid (D) at position 23 to lysine (K) at position 129.

DNAs encoding the 125M10AA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125M10AA heavy chain nucleic acid sequence> (SEQ ID NO: 26)

10 20 30 40 50 60

ATGGATCTCA TGTGCAAGAA AATGAAGCAC CTGTGGTTCT TCCTCCTGCT GGTGGCGGCT
 70 80 90 100 110 120
 CCCAGATGGG TCCTGTCCCA GCTGCAGGTG CAGGAGTCGG GCCCAGGACT GGTGAAGCCT
 130 140 150 160 170 180
 TCGGAGACCC TGTCCCTCAT CTGCACTGTC TCTGGTGGCT CCATCAGGAC CAGTGGTTAC
 190 200 210 220 230 240
 TACTGGGGCT GGTTCGCCA GCCCCCAGGG AAGGGACTGG AGTGGATTGG GACTAGTCAT
 250 260 270 280 290 300
 AATAGTGGGA GCACCTACTA CAACCCGTCC CTCAAGAGTC GAGTCACCAT ATCCGTAGAC
 310 320 330 340 350 360
 ACGTCCAAGA ACCAGTTCTC CCTGAAGCTG AACTCTGTGA CCGCCGCAGA CACGGCTGTG
 370 380 390 400 410 420
 TATTACTGTG CGAGACAAGG TTACGATTTT AAAGTCAATA TAGACGTCTG GGGACAAGGG
 430 440 450
 ACCACGGTCA CCGTCTCCTC AGCTAGC...

<125M10AA heavy chain amino acid sequence> (SEQ ID NO: 27)

10 20 30 40 50 60
 MDLMCKMKH LWFFLLLVA PRWVLSQLQV QESGPGLVKP SETLSLICTV SGGSIRTSGY
 70 80 90 100 110 120
 YGWFRQPPG KGLEWIGTSH NSGSTYYNPS LKSRVTISVD TSKNQFSLKL NSVTAADTAV
 130 140 150
 YYCARQGYDF KVNIDVWGQG TTVTVSSAS.

<125M10AA light chain nucleic acid sequence> (SEQ ID NO: 28)

10 20 30 40 50 60
 ATGGAAGCCC CAGCTCAGCT TCTCTTCCTC CTGCTACTCT GGCTCCCAGA TACCACCGGA
 70 80 90 100 110 120
 GAAATTGTGT TGACACAGTC TCCAGCCACC CTGTCTTTGT CTCCAGGGGA AAGAGCCACC
 130 140 150 160 170 180
 CTCTCCTGCA GGGCCAGTCA GAGTGTTAGC AGCTACTTAG CCTGGTACCA ACAGAAACCT

```

      190      200      210      220      230      240
GGCCAGGCTC CCAGGCTCCT CATCTATGAT GCATCCAACA GGGCCACTGG CATCCCAGCC
      250      260      270      280      290      300
AGGTTCAGTG GCAGTGGGTC TGGGACAGAC TTCACTCTCA CCATCAGCAG CCTAGAGCCT
      310      320      330      340      350      360
GAAGATTTTG CAGTTTATTA CTGTCAGCAG CGTAGCAACT GGCCGCTCAC TTTCGGCGGA
      370      380      390
GGGACCAAGG TGGAGATCAA ACGA.....

```

<125M10AA light chain amino acid sequence> (SEQ ID NO: 29)

```

      10      20      30      40      50      60
MEAPAQLLFL LLLWL PDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP
      70      80      90     100     110     120
GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPLTFGG
      130
GTKVEIKR..

```

In the heavy chain nucleic acid sequence (SEQ ID NO: 26), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 441 and guanine (G) at position 442. In the heavy chain amino acid sequence (SEQ ID NO: 27), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 147 and alanine (A) at position 148. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 26), the boundary between the signal sequence and the antibody variable region is located between cytosine (C) at position 78 and cytosine (C) at position 79. In the heavy chain amino acid sequence (SEQ ID NO: 27), the boundary between the signal sequence and the antibody variable region is located between serine (S) at position 26 and glutamine (Q) at position 27.

Accordingly, the variable region in the 125M10AA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 26) ranging from cytosine (C) at position 79 to adenine (A) at position 441. Further, the variable region in the heavy chain has the

amino acid sequence (SEQ ID NO: 27) ranging from glutamine (Q) at position 27 to serine (S) at position 147.

In the light chain nucleic acid sequence (SEQ ID NO: 28), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 381 and cytosine (C) at position 382. In the light chain amino acid sequence (SEQ ID NO: 29), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 127 and arginine (R) at position 128. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 28), the boundary between the signal sequence and the antibody variable region is located between adenine (A) at position 60 and guanine (G) at position 61. In the light chain amino acid sequence (SEQ ID NO: 29), the boundary between the signal sequence and the antibody variable region is located between glycine (G) at position 20 and glutamic acid (E) at position 21.

Accordingly, the variable region in the 125M10AA antibody light chain has the nucleic acid sequence (SEQ ID NO: 28) ranging from guanine (G) at position 61 to adenine (A) at position 381. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 29) ranging from glutamic acid (E) at position 21 to lysine (K) at position 127.

DNAs encoding the 125M165DAAA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125M165DAAA heavy chain nucleic acid sequence> (SEQ ID NO: 30)

10	20	30	40	50	60
ATGGAGTTTG	GGCTGAGCTG	GGTTTTCCTC	GTTGCTCTTT	TAAGAGGTGT	CCAGTGTCAG
70	80	90	100	110	120
GTGCAGCTGG	TGGAGTCTGG	GGGAGGCGTG	GTCCAGCCTG	GGAGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCGT	CTGGATTAC	CTTCAGTTAT	TATGGCATGC	ACTGGGTCCG	CCAGGCTCCA
190	200	210	220	230	240

GGCAAGGGGC TGGAGTGGGT GGCAGTTATA TGGTATGATG GAAGTAATAA ATACTATGCA
 250 260 270 280 290 300
 GACTCCGTGA AGGGCCGATT CACCATCTCC AGAGACAATT CCAAGAAAAC GCTGTATCTG
 310 320 330 340 350 360
 CAAATGAACA GCCTGAGAGC CGAGGACACG GCTGTGTATT ACTGTGCGAG AGATGGGCAT
 370 380 390 400 410 420
 AGCAGTGGCT GGGGGGACTT CCAGCACTGG GGCCAGGGCA CCCTGGTCAC CGTCTCCTCA
 430
 GCTAGC. . . .

<125M165DAAA heavy chain amino acid sequence> (SEQ ID NO: 31)

10 20 30 40 50 60
 MEFGLSWVFL VALLRGVQCQ VQLVESGGGV VQPGRSLRLS CAASGFTFSY YGMHWVRQAP
 70 80 90 100 110 120
 GKGLEWVAVI WYDGSNKYYA DSVKGRFTIS RDNSKKTLYL QMNSLRAEDT AVYYCARDGH
 130 140 150
 SSGWGDFQHW GQGTTLTVSS AS.

<125M165DAAA light chain nucleic acid sequence> (SEQ ID NO: 32)

10 20 30 40 50 60
 ATGGAAGCCC CAGCTCAGCT TCTCTTCCTC CTGCTACTCT GGCTCCCAGA TACCACCGGA
 70 80 90 100 110 120
 GAAATTGTGT TGACACAGTC TCCAGCCACC CTGTCTTTGT CTCCAGGGGA AAGAGCCACC
 130 140 150 160 170 180
 CTCTCCTGCA GGGCCAGTCA GAGTGTTAGC AGCTCCTTAG CCTGGTACCA ACAGAAACCT
 190 200 210 220 230 240
 GGCCAGGCTC CCAGGCTCCT CATCTATGAT GCATCCAACA GGGCCACTGG CATCCCAGCC
 250 260 270 280 290 300
 AGGTTTCAGTG GCAGTGGGTC TGGGACAGAC TTCACTCTCA CCATCAGCAG CCTAGAGCCT
 310 320 330 340 350 360
 GAAGATTTTG CAATTTATTA CTGTCAGCAG CGTAGCAACT GGCCTCCGAC GTTCGGCCAA

370 380 390
 GGGACCAAGG TGGAAATCAA ACGA.....
 <125M165DAAA light chain amino acid sequence> (SEQ ID NO: 33)
 10 20 30 40 50 60
 MEAPAQLLFL LLLWLPTDTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SSLAWYQQKP
 70 80 90 100 110 120
 GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAIYYCQQ RSNWPPTFGQ
 130
 GTKVEIKR..

In the heavy chain nucleic acid sequence (SEQ ID NO: 30), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy chain amino acid sequence (SEQ ID NO: 31), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 30), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and cytosine (C) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 31), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamine (Q) at position 20.

Accordingly, the variable region in the 125M165DAAA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 30) ranging from cytosine (C) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 31) ranging from glutamine (Q) at position 20 to serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 32), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 381 and cytosine (C) at position 382. In the light chain amino acid sequence (SEQ ID NO: 33), the boundary between the antibody variable region and the

antibody constant region is located between lysine (K) at position 127 and arginine (R) at position 128. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 32), the boundary between the signal sequence and the antibody variable region is located between adenine (A) at position 60 and guanine (G) at position 61. In the light chain amino acid sequence (SEQ ID NO: 33), the boundary between the signal sequence and the antibody variable region is located between glycine (G) at position 20 and glutamic acid (E) at position 21.

Accordingly, the variable region in the 125M165DAAA antibody light chain has the nucleic acid sequence (SEQ ID NO: 32) ranging from guanine (G) at position 61 to adenine (A) at position 381. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 33) ranging from glutamic acid (E) at position 21 to lysine (K) at position 127.

DNAs encoding the 125M96ABA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125M96ABA heavy chain nucleic acid sequence> (SEQ ID NO: 34)

10	20	30	40	50	60
ATGAAGCACC	TGTGGTTCTT	CCTCCTGCTG	GTGGCGGCTC	CCAGATGGGT	CCTGTCCCAA
70	80	90	100	110	120
CTGCAGCTGC	AGGAGTCGGG	CCCAGGACTG	GTGAAGCCTT	CGGAGACCCT	GTCCCTCACC
130	140	150	160	170	180
TGCACTGTCT	CTGGTGGCTC	CATCAGCACT	AGTAGTTACT	ACTGGGGCTG	GATCCGCCAG
190	200	210	220	230	240
CCCCCGGGA	AGGGCCTGGA	ATGGATTGGG	ACTATCTATT	ATAATGGGAG	CACCTACTAC
250	260	270	280	290	300
AGCCCGTCCC	TCAAGAGTCG	AGTCAGTATA	TCCGTAGACA	CGTCCAAGAA	CCAGTTCTCC
310	320	330	340	350	360
CTGAAGCTGA	GCTCTGTGAC	CGCCGCAGAC	ACGTCTGTGT	ATTACTGTGC	GAGACAAGGT
370	380	390	400	410	420

TACGATATTA AAATCAATAT AGACGTCTGG GGCCAAGGGA CCACGGTCAC CGTCTCCTCA
430

GCTAGC....

<125M96ABA heavy chain amino acid sequence> (SEQ ID NO: 35)

10	20	30	40	50	60
MKHLWFFLL	VAAPRWVLSQ	LQLQESGPGL	VKPSETLSLT	CTVSGGSIST	SSYYWGWIRQ
70	80	90	100	110	120
PPGKGLEWIG	TIYYNGSTYY	SPSLKSRVSI	SVDTSKNQFS	LKLSSVTAAD	TSVYYCARQG
130	140	150			
YDIKINIDVW	GQGTTVTVSS	AS.....			

<125M96ABA light chain nucleic acid sequence> (SEQ ID NO: 36)

10	20	30	40	50	60
ATGGAAGCCC	CAGCTCAGCT	TCTCTTCCTC	CTGCTACTCT	GGCTCCCAGA	TACCACCGGA
70	80	90	100	110	120
GAAATTGTGT	TGACACAGTC	TCCAGCCACC	CTGTCTTTGT	CTCCAGGGGA	AAGAGCCACC
130	140	150	160	170	180
CTCTCCTGCA	GGGCCAGTCA	GAGTGTTAGC	AGCTACTTAG	CCTGGTACCA	ACAGAAACCT
190	200	210	220	230	240
GGCCAGGCTC	CCAGGCTCCT	CATCTATGTT	GCATCCAACA	GGGCCACTGG	CATCCCAGCC
250	260	270	280	290	300
AGGTTTCAGTG	GCAGTGGGTC	TGGGACAGAC	TTCACTCTCA	CCATCAGCAG	CCTAGAGCCT
310	320	330	340	350	360
GAAGATTTTG	CAGTTTATTA	CTGTCAGCAG	CGTAGCAACT	GGCCGCTCAC	TTTCGGCGGA
370	380	390			
GGGACCAAGG	TGGAGATCAA	ACGA.....			

<125M96ABA light chain amino acid sequence> (SEQ ID NO: 37)

10	20	30	40	50	60
MEAPAQLLFL	LLLWLPDTTG	EIVLTQSPAT	LSLSPGERAT	LSCRASQSVS	SYLAWYQQKP
70	80	90	100	110	120

GQAPRLLIYV ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPLTFGG

130

GTKVEIKR. .

In the heavy chain nucleic acid sequence (SEQ ID NO: 34), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy chain amino acid sequence (SEQ ID NO: 35), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 34), the boundary between the signal sequence and the antibody variable region is located between cytosine (C) at position 57 and cytosine (C) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 35), the boundary between the signal sequence and the antibody variable region is located between serine (S) at position 19 and glutamine (Q) at position 20.

Accordingly, the variable region in the 125M96ABA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 34) ranging from cytosine (C) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 35) ranging from glutamine (Q) at position 20 to serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 36), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 381 and cytosine (C) at position 382. In the light chain amino acid sequence (SEQ ID NO: 37), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 127 and arginine (R) at position 128. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 36), the boundary between the signal sequence and the antibody variable region is located between adenine (A) at position 60 and guanine (G) at position 61. In the light chain amino acid sequence (SEQ ID NO: 37), the boundary between the signal sequence and the antibody variable region is located between glycine (G) at position 20 and glutamic

acid (E) at position 21.

Accordingly, the variable region in the 125M165DAAA antibody light chain has the nucleic acid sequence (SEQ ID NO: 36) ranging from guanine (G) at position 61 to adenine (A) at position 381. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 37) ranging from glutamic acid (E) at position 21 to lysine (K) at position 127.

DNAs encoding the 125N26F6AA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125N26F6AA heavy chain nucleic acid sequence> (SEQ ID NO: 38)

10	20	30	40	50	60
ATGGAGTTTG	GGCTGAGCTG	GGTTTTCTC	GTTGCTCTTT	TAAGAGGTGT	CCAGTGTCAG
70	80	90	100	110	120
GTGCAGTTGG	TGGAGTCTGG	GGGAGGCGTG	GTCCAGCCTG	GGAGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCGT	CTGGATTAC	CTTCAGTCAC	TATGGCATGC	ACTGGGTCCG	CCAGGCTCCA
190	200	210	220	230	240
GGCAAGGGGC	TGGAGTGGGT	GGCACTTATA	TGGTATGATG	GAAGTAATAA	ATACTATGCA
250	260	270	280	290	300
GACTCCGTGA	AGGGCCGATT	CACCATCTCC	AGAGACAATT	CCAAGAACAC	GCTGTATCTG
310	320	330	340	350	360
CAAATGAACA	GCCTGAGAGC	CGAGGACACG	GCTGTGTATT	ACTGTGCGAG	AGATCCCTTA
370	380	390	400	410	420
GCAGCTGGTA	CGTCCTACTT	TGACTACTGG	GGCCAGGGAA	CCCTGGTCAC	CGTCTCCTCA
430					
GCTAGC...					

<125N26F6AA heavy chain amino acid sequence> (SEQ ID NO: 39)

10	20	30	40	50	60
MEFGLSWVFL	VALLRGVQCQ	VQLVESGGGV	VQPGRSLRLS	CAASGFTFSH	YGMHWVRQAP

70 80 90 100 110 120
 GKGLEWVALI WYDGSNKYYA DSVKGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARDPL
 130 140 150
 AAGTSYFDYW GQGTLVTVSS AS.....

<125N26F6AA light chain nucleic acid sequence> (SEQ ID NO: 40)

10 20 30 40 50 60
 ATGTCGCCAT CACAACATCAT TGGGTTTCTG CTGCTCTGGG TTCCAGCCTC CAGGGGTGAA
 70 80 90 100 110 120
 ATTGTGCTGA CTCAGTCTCC AGACTTTCAG TCTGTGACTC CAAAGGAGAA AGTCACCATC
 130 140 150 160 170 180
 ACCTGCCGGG CCAGTCAGAG CATTGGTAGT AGCTTACACT GGTACCAGCA GAAACCAGAT
 190 200 210 220 230 240
 CAGTCTCCAA AGCTCCTCAT CAAGTATGCT TCCCAGTCCT TCTCAGGGGT CCCCTCGAGG
 250 260 270 280 290 300
 TTCAGTGGCA GTGGATCTGG GACAGATTTC ACCCTCACCA TCAATAGCCT GGAAGCTGAA
 310 320 330 340 350 360
 GATGCTGCAG CGTATTACTG TCATCAGAGT AGTAGTTTAC CATTCACTTT CGGCCCTGGG
 370 380
 ACCAAAGTGG ATATCAAACG A

<125N26F6AA light chain amino acid sequence> (SEQ ID NO: 41)

10 20 30 40 50 60
 MSPSQLIGFL LLWVPASRGE IVLTQSPDFQ SVTPKEKVTI TCRASQSIGS SLHWYQQKPD
 70 80 90 100 110 120
 QSPKLLIKYA SQSFSGVPSR FSGSGSGTDF TLTINSLEAE DAAAYYCHQS SSLPFTFGPG
 130
 TKVDIKR...

In the heavy chain nucleic acid sequence (SEQ ID NO: 38), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy

chain amino acid sequence (SEQ ID NO: 39), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 38), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and cytosine (C) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 39), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamine (Q) at position 20.

Accordingly, the variable region in the 125M96ABA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 38) ranging from cytosine (C) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 39) ranging from glutamine (Q) at position 20 to serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 40), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 388 and cytosine (C) at position 389. In the light chain amino acid sequence (SEQ ID NO: 41), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 126 and arginine (R) at position 127. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 40), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position 58. In the light chain amino acid sequence (SEQ ID NO: 41), the boundary between the signal sequence and the antibody variable region is located between glycine (G) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the 125M96ABA antibody light chain has the nucleic acid sequence (SEQ ID NO: 40) ranging from guanine (G) at position 58 to adenine (A) at position 388. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 41) ranging from glutamic acid (E) at position 20 to lysine (K) at position 126.

DNAs encoding the 125Q47BA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125Q47BA heavy chain nucleic acid sequence> (SEQ ID NO: 42)

10	20	30	40	50	60
ATGGAGTTTG	GGCTGAGCTG	GCTTTTCTT	GTGGCTATTT	TAAAAGGTGT	CCAGTGTGAG
70	80	90	100	110	120
GTGCAGCTGT	TGGAGTCTGG	GGGAGGCTTG	GTACAGCCTG	GGGGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCCT	CTGGATTAC	CTTTAGCAGC	TATGCCATGA	GCTGGGTCCG	CCAGGCTCCA
190	200	210	220	230	240
GGGAAGGGGC	TGGAGTGGGT	CTCAGATATT	AGTGGTAGTG	GTGGTTACAC	ATACTACGCA
250	260	270	280	290	300
GACTCCGTGA	AGGGCCGGTT	CACCATCTCC	AGAGACAATT	CCAAGAACAC	GCTGTATCTG
310	320	330	340	350	360
CAAATGAACA	GCCTGAGAGC	CGAGGACACG	GCCGTATATT	ACTGTGCGAA	AACAGGCGAT
370	380	390	400	410	420
GGTTCGGGGA	GTTATTCCCC	TGACTCCTGG	GGCCAGGGAA	CCCTGGTCAC	CGTCTCCTCA
430					
GCTAGC.	...				

<125Q47BA heavy chain amino acid sequence> (SEQ ID NO: 43)

10	20	30	40	50	60
MEFGLSWLFL	VAILKGVQCE	VQLLESGGGL	VQPGGSLRLS	CAASGFTFSS	YAMTWVRQAP
70	80	90	100	110	120
GKGLEWVSDI	SGSGGYTYYA	DSVKGRFTIS	RDNSKNTLYL	QMNSLRAEDT	AVYYCAKTGA
130	140	150			
GSGSYSPDSW	GQGTLLTVSS	AS.			

<125Q47BA light chain nucleic acid sequence> (SEQ ID NO: 44)

10	20	30	40	50	60
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ATGGACATGA GGGTCCTCGC TCAGCTCCTG GGGCTCCTGC TGCTCTGTTT CCCAGGTGCC
 70 80 90 100 110 120
 AGATGTGACA TCCAGATGAC CCAGTCTCCA TCCTCACTGT CTGCATCTGT AGGAGACAGA
 130 140 150 160 170 180
 GTCACCATCA CTTGTCGGGC GAGTCAGGGT ATTAGCAGCT GGTTAGCCTG GTATCAGCAG
 190 200 210 220 230 240
 AAACCAGAGA AAGCCCCTAA GTCCCTGATC TATGCTGCAT CCAGTTTGCA AAGTGGGGTC
 250 260 270 280 290 300
 CCATCAAGGT TCAGCGGCAG TGGATCTGGG ACAGATTTC A CTCTCACCAT CAGCAGCCTG
 310 320 330 340 350 360
 CAGCCTGAAG ATTTTGCAAC TTATTACTGC CAACAGTATA ATAGTTACCC GTACACTTTT
 370 380 390
 GGCCAGGGGA CCAAGCTGGA GATCAAACGA

<125Q47BA light chain amino acid sequence> (SEQ ID NO: 45)

10 20 30 40 50 60
 MDMRVLAQLL GLLLLCFPGA RCDIQMTQSP SSLASVGDR VTITCRASQG ISSWLAWYQQ
 70 80 90 100 110 120
 KPEKAPKSLI YAASSLQSGV PSRFSGSGSG TDFTLTISL QPEDFATYYC QQYNSYPYTF
 130
 GQGTKLEIKR

In the heavy chain nucleic acid sequence (SEQ ID NO: 42), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy chain amino acid sequence (SEQ ID NO: 43), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 42), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 43), the boundary between

the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the 125Q47BA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 42) ranging from guanine (G) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 43) ranging from glutamic acid (E) at position 20 to serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 44), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 387 and cytosine (C) at position 388. In the light chain amino acid sequence (SEQ ID NO: 45), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 129 and arginine (R) at position 130. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 44), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 66 and guanine (G) at position 67. In the light chain amino acid sequence (SEQ ID NO: 45), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 22 and aspartic acid (D) at position 23.

Accordingly, the variable region in the 125Q47BA antibody light chain has the nucleic acid sequence (SEQ ID NO: 44) ranging from guanine (G) at position 67 to adenine (A) at position 387. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 45) ranging from aspartic acid (D) at position 23 to lysine (K) at position 129.

DNAs encoding the 125Q54AAAA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125Q54AAAA heavy chain nucleic acid sequence> (SEQ ID NO: 46)

10	20	30	40	50	60
ATGGAGTTTG GGCTGAGCTG GCTTTTCTT GTGGCTATTT TAAAGGTGT CCAGTGTGAG					

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      70          80          90          100          110          120
GTGCAGCTGT TGGAGTCTGG GGGAGGCTTG GTACAGCCTG GGGGGTCCCT GAGACTCTCC

      130          140          150          160          170          180
TGTGCAGCCT CTGGATTCAC CTTTAGCAGC TATGCCATGA GCTGGGTCCG CCAGGCTCCA

      190          200          210          220          230          240
GGGAAGGGGC TGGAGTGGGT CTCAGATATT AGTGGTAGTG GTGGTTACAC ATACTACGCA

      250          260          270          280          290          300
GACTCCGTGA AGGGCCGGTT CACCATCTCC AGAGACAATT CCAAGAACAC GCTGTATCTG

      310          320          330          340          350          360
CAAATGAACA GCCTGAGAGC CGAGGACACG GCCGTATATT ACTGTGCGAA AACAGGCGAT

      370          380          390          400          410          420
GGTTCGGGGA GTTATTCCCC TGA CTCCTGG GCCAGGGAA CCCTGGTCAC CGTCTCCTCA

      430

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GCTAGC. . . .

<125Q54AAAA heavy chain amino acid sequence> (SEQ ID NO: 47)

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      10          20          30          40          50          60
MEFGLSWLFL VAILKGVQCE VQLLESGGGL VQPGGSLRLS CAASGFTFSS YAMSWVRQAP

      70          80          90          100          110          120
GKGLEWVSDI SGSGGYTYYA DSVKGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCAKTGD

      130          140          150
GSGSYSPDSW GQGLVTVSS AS. . . . .

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<125Q54AAAA light chain nucleic acid sequence> (SEQ ID NO: 48)

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      10          20          30          40          50          60
ATGGACATGA GGGTCCTCGC TCAGCTCCTG GGGTCCTGC TGCTCTGTTT CCCAGGTGCC

      70          80          90          100          110          120
AGATGTGACA TCCAGATGAC CCAGTCTCCA TCCTCACTGT CTGCATCTGT AGGAGACAGA

      130          140          150          160          170          180
GTCACCATCA CTTGTCGGGC GAGTCAGGGT ATTAGCAGGT GGTTAGCCTG GTATCAGCAG

      190          200          210          220          230          240

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AAACCAGAGA AAGCCCCTAA GTCCCTGATC TATGCTGCAT CCAGTTTGCA AAGTGGGGTC
      250      260      270      280      290      300
CCATCAAGGT TCAGCGGCAG TGGATCTGGG ACAGATTTC A CTCTACCAT CAGCAGCCTG
      310      320      330      340      350      360
CAGCCTGAAG ATTTTGCAAC TTATTACTGC CAACAGTATA ATAGTTACCC GTACACTTTT
      370      380      390
GGCCAGGGGA CCAAGCTGGA GATCAAACGA

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<125Q54AAAA light chain amino acid sequence> (SEQ ID NO: 49)

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      10      20      30      40      50      60
MDMRVLAQLL GLLLLCFPGA RCDIQMTQSP SSSLASVGDR VTITCRASQG ISRWLAWYQQ
      70      80      90     100     110     120
KPEKAPKSLI YAASSLQSGV PSRFSGSGSG TDFTLTISL QPEDFATYYC QQYNSYPYTF
      130
GQGTKLEIKR

```

In the heavy chain nucleic acid sequence (SEQ ID NO: 46), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy chain amino acid sequence (SEQ ID NO: 47), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 46), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 47), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the 125Q54AAAA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 46) ranging from guanine (G) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 47) ranging from glutamic acid (E) at position 20 to

serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 48), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 387 and cytosine (C) at position 388. In the light chain amino acid sequence (SEQ ID NO: 49), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 129 and arginine (R) at position 130. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 48), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 66 and guanine (G) at position 67. In the light chain amino acid sequence (SEQ ID NO: 49), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 22 and aspartic acid (D) at position 23.

Accordingly, the variable region in the 125Q54AAAA antibody light chain has the nucleic acid sequence (SEQ ID NO: 48) ranging from guanine (G) at position 67 to adenine (A) at position 387. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 49) ranging from aspartic acid (D) at position 23 to lysine (K) at position 129.

DNAs encoding the 125R5AAAA heavy chain variable region and the light chain variable region and the amino acid sequences of the heavy chain variable region and the light chain variable region are each shown below.

<125R5AAAA heavy chain nucleic acid sequence> (SEQ ID NO: 50)

10	20	30	40	50	60
ATGGAGTTTG	GGCTGAGCTG	GCTTTTTCTT	GTGGCTATTT	TAAAAGGTGT	CCAGTGTGAG
70	80	90	100	110	120
GTGCAGCTGT	TGGAGTCTGG	GGGAGGCTTG	GTACAGCCTG	GGGGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCCT	CTGGATTCAC	CTTTAGCAGC	TATGCCATGA	GCTGGGTCCG	CCAGGCTCCA
190	200	210	220	230	240
GGGAAGGGGC	TGGAGTGGGT	CTCAGATATT	AGTGGTAGTG	GTGGTTACAC	ATACTACGCA

250 260 270 280 290 300
 GACTCCGTGA AGGGCCGGTT CACCATCTCC AGAGACAATT CCAAGAAAAC GCTGTATCTG
 310 320 330 340 350 360
 CAAATGAACA GCCTGAGAGC CGAGGACACG GCCGTATATT ACTGTGCGAA AACAGGCGAT
 370 380 390 400 410 420
 GGTTCGGGGA GTTATTCCCC TGACTACTGG GGCCAGGGAA CCCTGGTCAC CGTCTCCTCA
 430

GCTAGC. . . .

<125R5AAAA heavy chain amino acid sequence> (SEQ ID NO: 51)

10 20 30 40 50 60
 MEFGLSWLFL VAILKGVQCE VQLLESGGGL VQPGGSLRLS CAASGFTFSS YAMSWVRQAP
 70 80 90 100 110 120
 GKGLEWVSDI SGSGGYTYYA DSVKGRFTIS RDNSKKTLYL QMNSLRAEDT AVYYCAKTGD
 130 140 150
 GSGSYSPDYW GQGT LVT VSS AS.

<125R5AAAA light chain nucleic acid sequence> (SEQ ID NO: 52)

10 20 30 40 50 60
 ATGGACATGA GGGTCCTCGC TCAGCTCCTG GGGCTCCTGC TGCTCTGTTT CCCAGGTGCC
 70 80 90 100 110 120
 AGATGTGACA TCCAGATGAC CCAGTCTCCA TCCTCACTGT CTGCATCTGT AGGAGACAGA
 130 140 150 160 170 180
 GTCACCATCA CTTGTCGGGC GAGTCAGGGT ATTAGCAGCT GGTTAGCCTG GTATCAGCAG
 190 200 210 220 230 240
 AAACCAGAGA AAGCCCCTAA GTCCCTGATC TATGCTGCAT CCAGTTTGCA AAGTGGGGTC
 250 260 270 280 290 300
 CCATCAAGGT TCAGCGGCAG TGGATCTGGG ACAGATTTC A CTCTACCAT CAGCAGCCTG
 310 320 330 340 350 360
 CAGCCTGAAG ATTTTGCAAC TTATTACTGC CAACAGTATA ATAGTTACCC GTACACTTTT
 370 380 390

GGCCAGGGGA CCAAGCTGGA GATCAAACGA

<125R5AAAA light chain amino acid sequence> (SEQ ID NO: 53)

10	20	30	40	50	60
MDMRVLAQLL GLLLLCFPGA RCDIQMTQSP SLSASVGDR VTITCRASQG ISSWLAWYQQ					
70	80	90	100	110	120
KPEKAPKSLI YAASSLQSGV PSRFGSGSG TDFTLTISL QPEDFATYYC QQYNSYPYTF					
130					
GQGTKLEIKR					

In the heavy chain nucleic acid sequence (SEQ ID NO: 50), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 420 and guanine (G) at position 421. In the heavy chain amino acid sequence (SEQ ID NO: 51), the boundary between the antibody variable region and the antibody constant region is located between serine (S) at position 140 and alanine (A) at position 141. Furthermore, in the heavy chain nucleic acid sequence (SEQ ID NO: 50), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 57 and guanine (G) at position 58. In the heavy chain amino acid sequence (SEQ ID NO: 51), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 19 and glutamic acid (E) at position 20.

Accordingly, the variable region in the 125R5AAAA antibody heavy chain has the nucleic acid sequence (SEQ ID NO: 50) ranging from guanine (G) at position 58 to adenine (A) at position 420. Further, the variable region in the heavy chain has the amino acid sequence (SEQ ID NO: 51) ranging from glutamic acid (E) at position 20 to serine (S) at position 140.

In the light chain nucleic acid sequence (SEQ ID NO: 52), the boundary between the antibody variable region and the antibody constant region is located between adenine (A) at position 387 and cytosine (C) at position 388. In the light chain amino acid sequence (SEQ ID NO: 53), the boundary between the antibody variable region and the antibody constant region is located between lysine (K) at position 129 and arginine (R)

at position 130. Furthermore, in the light chain nucleic acid sequence (SEQ ID NO: 52), the boundary between the signal sequence and the antibody variable region is located between thymine (T) at position 66 and guanine (G) at position 67. In the light chain amino acid sequence (SEQ ID NO: 53), the boundary between the signal sequence and the antibody variable region is located between cysteine (C) at position 22 and aspartic acid (D) at position 23.

Accordingly, the variable region in the 125R5AAAA antibody light chain has the nucleic acid sequence (SEQ ID NO: 52) ranging from guanine (G) at position 67 to adenine (A) at position 387. Further, the variable region in the light chain has the amino acid sequence (SEQ ID NO: 53) ranging from aspartic acid (D) at position 23 to lysine (K) at position 129.

Example 14 Determination of full-length sequences containing constant regions of human antibody heavy chain and light chain genes expressed by hybridomas 125N26F6AA and 125M10AA

The DNA nucleotide sequence and the amino acid sequence of the antibody variable region of each antibody were determined in Example 13. For the hybridomas 125N26F6AA and 125M10AA derived from KM mice, full-length sequences containing constant regions were analyzed. cDNA synthesis was performed according to Example 13 using as materials total RNAs prepared from the hybridomas 125N26F6AA and 125M10AA and a SMART RACE cDNA amplification Kit (produced by Becton Dickinson Bioscience Clontech).

1st strand cDNA synthesis

Total RNA	5 µg/3 µl
3'CDS primer	1 µl
H ₂ O	1 µl

A reaction solution with the above composition was incubated at 70°C for 2 minutes and then the following components were added, followed by 1.5 hours of incubation at 42°C.

5 × Buffer	2 µl
DTT	1 µl
dNTP mix	1 µl
PowerScript Reverse Transcriptase	1 µl

Furthermore, 50 µl of a tricine buffer was added and then incubation was performed at 72°C for 7 minutes, thereby obtaining 1st strand cDNA.

To obtain a DNA containing the entire coding region of a constant region, a PCR amplification reaction was performed using the above synthesized cDNA as a template and a primer set of: a synthetic DNA (5' primer) having a sequence binding around the ATG initiation codon at the 5' end of each antibody gene; and a synthetic DNA (3' primer) specifically binding to the 3' non-translation region of a human antibody gene. As a result of the amplification reaction, the full-length sequence of an antibody gene (cDNA) ranging from the ATG initiation codon to the 3' non-translation region, including the stop codon, could be obtained.

The 125N26F6AA heavy chain DNA was amplified by repeating an incubation cycle (94°C for 15 seconds and 68°C for 2 minutes) 35 times using a primer set of an H chain 5' primer: N26H5Sal1 (SEQ ID NO: 58) and an H chain 3' primer: H_3UTR1848 (5'-CGGGGTACGTGCCAAGCATCCTCGTG-3', SEQ ID NO: 74) or a primer set of the H chain 5' primer: N26H5Sal1 (SEQ ID NO: 58) and an H chain 3' primer: H_3UTR1875 (5'-ATGCTGGGCGCCCGGGAAGTATGTAC-3', SEQ ID NO: 75). Meanwhile, the 125N26F6AA light chain (κ) was amplified using a primer set of an L chain 5' primer: N26KA10 Minor L Bgl (SEQ ID NO: 64) and an L chain 3' primer: L_3UTR_823 (5'-GAAAGATGAGCTGGAGGACCGCAATA-3', SEQ ID NO: 76). Except for primers, amplification was performed with a reaction solution having the same composition as that employed in Example 13 (2)-1.

For amplification of the 125M10AA heavy chain DNA, a primer set of an H chain 5' primer: M10H5Sal (SEQ ID NO: 70) and the H chain 3' primer: H_3UTR1848 (SEQ ID NO: 74) or a primer set of the H chain 5' primer: M10H5Sal (SEQ ID NO: 70)

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and the H chain 3' primer: H_3UTR1875 (SEQ ID NO: 75) was used. For amplification of the 125M10AA light chain (κ) DNA, a primer set of an L chain 5' primer: M10KBgl (SEQ ID NO: 66) and the L chain 3' primer: L_3UTR_823 (SEQ ID NO: 76) were used.

The amplified PCR fragments were separately harvested by ethanol precipitation, harvested by agarose gel electrophoresis, and then purified using a QIAquick Gel Extraction Kit (produced by QIAGEN). The thus purified amplified fragments were separately subcloned into a PCR 4 Blunt-TOPO vector of a Zero Blunt TOPO PCR Cloning Kit (produced by Invitrogen). For the thus obtained clones, sequencing template DNAs were prepared using a TempliPhi DNA Amplification Kit (produced by Amersham Biosciences) including reagents for preparation of sequencing template DNA according to the protocols included therein. Thus, the nucleotide sequences of the insert DNAs were determined. Primers used for analyses of the DNA nucleotide sequences of the human antibody heavy chains were M13FW (SEQ ID NO: 3), M13RV (SEQ ID NO: 4), hh4 (SEQ ID NO: 21), hh1 (5'-CCAAGGGCCCATCGGTCTTCCCCCTGGCAC-3') (SEQ ID NO: 77), CMVH903F (5'-GACACCCTCATGATCTCCCGGACC-3') (SEQ ID NO: 78), CMVHR1303 (5'-TGTTCTCCGGCTGCCCATTGCTCT-3') (SEQ ID NO: 79), hh-6 (5'-GGTCCGGGAGATCATGAGGGTGTCTT-3') (SEQ ID NO: 80), hh-2 (SEQ ID NO: 20), H_3UTR1848 (SEQ ID NO: 74), and H_3UTR1875 (SEQ ID NO: 75). Primers used for analyses of the DNA nucleotide sequences of the human antibody light chains (κ) were M13FW (SEQ ID NO: 3), M13RV (SEQ ID NO: 4), hk-5 (SEQ ID NO: 19), and hk-1 (5'-TGGCTGCACCATCTGTCTTCATCTTC-3') (SEQ ID NO: 81).

DNAs encoding the entire 125N26F6AA antibody heavy chain region and the entire light chain region and the amino acid sequences of the entire heavy chain region and the entire light chain region are each shown below.

<125N26F6AA heavy chain nucleic acid sequence> (SEQ ID NO: 82)

10 20 30 40 50 60

*Trade-mark

ATGGAGTTTG	GGCTGAGCTG	GGTTTTCCTC	GTTGCTCTTT	TAAGAGGTGT	CCAGTGTCAG
70	80	90	100	110	120
GTGCAGTTGG	TGGAGTCTGG	GGGAGGCGTG	GTCCAGCCTG	GGAGGTCCCT	GAGACTCTCC
130	140	150	160	170	180
TGTGCAGCGT	CTGGATTAC	CTTCAGTCAC	TATGGCATGC	ACTGGGTCCG	CCAGGCTCC
A 190	200	210	220	230	240
GGCAAGGGGC	TGGAGTGGGT	GGCACTTATA	TGGTATGATG	GAAGTAATAA	ATACTATGCA
250	260	270	280	290	300
GACTCCGTGA	AGGGCCGATT	CACCATCTCC	AGAGACAATT	CCAAGAACAC	GCTGTATCTG
310	320	330	340	350	360
CAAATGAACA	GCCTGAGAGC	CGAGGACACG	GCTGTGTATT	ACTGTGCGAG	AGATCCCTTA
370	380	390	400	410	420
GCAGCTGGTA	CGTCCTACTT	TGACTACTGG	GGCCAGGGAA	CCCTGGTCAC	CGTCTCCTCA
430	440	450	460	470	480
GCCTCCACCA	AGGGCCCATC	GGTCTTCCCC	CTGGCACCCCT	CCTCCAAGAG	CACCTCTGGG
490	500	510	520	530	540
GGCACAGCGG	CCCTGGGCTG	CCTGGTCAAG	GACTACTTCC	CCGAACCGGT	GACGGTGTCG
550	560	570	580	590	600
TGGA ACTCAG	GCGCCCTGAC	CAGCGGCGTG	CACACCTTCC	CGGCTGTCCT	ACAGTCCTCA
610	620	630	640	650	660
GGACTCTACT	CCCTCAGCAG	CGTGGTGACC	GTGCCCTCCA	GCAGCTTGGG	CACCCAGACC
670	680	690	700	710	720
TACATCTGCA	ACGTGAATCA	CAAGCCCAGC	AACACCAAGG	TGGACAAGAA	AGTTGAGCCC
730	740	750	760	770	780
AAATCTTGTG	ACAAA ACTCA	CACATGCCCA	CCGTGCCCAG	CACCTGAACT	CCTGGGGGGA
790	800	810	820	830	840
CCGTCAGTCT	TCCTCTTCCC	CCCAA AACCC	AAGGACACCC	TCATGATCTC	CCGGACCCCT
850	860	870	880	890	900
GAGGTCACAT	GCGTGGTGGT	GGACGTGAGC	CACGAAGACC	CTGAGGTCAA	GTTCAACTGG

910	920	930	940	950	960
TACGTGGACG	GCGTGGAGGT	GCATAATGCC	AAGACAAAGC	CGCGGGAGGA	GCAGTACAAC
970	980	990	1000	1010	1020
AGCACGTACC	GTGTGGTCAG	CGTCCTCACC	GTCCTGCACC	AGGACTGGCT	GAATGGCAAG
1030	1040	1050	1060	1070	1080
GAGTACAAGT	GCAAGGTCTC	CAACAAAGCC	CTCCCAGCCC	CCATCGAGAA	AACCATCTCC
1090	1100	1110	1120	1130	1140
AAAGCCAAAG	GGCAGCCCCG	AGAACCACAG	GTGTACACCC	TGCCCCCATC	CCGGGATGAG
1150	1160	1170	1180	1190	1200
CTGACCAAGA	ACCAGGTCAG	CCTGACCTGC	CTGGTCAAAG	GCTTCTATCC	CAGCGACATC
1210	1220	1230	1240	1250	1260
GCCGTGGAGT	GGGAGAGCAA	TGGGCAGCCG	GAGAACAAC	ACAAGACCAC	GCCTCCCGTG
1270	1280	1290	1300	1310	1320
CTGGACTCCG	ACGGCTCCTT	CTTCCTCTAC	AGCAAGCTCA	CCGTGGACAA	GAGCAGGTGG
1330	1340	1350	1360	1370	1380
CAGCAGGGGA	ACGTCTTCTC	ATGCTCCGTG	ATGCATGAGG	CTCTGCACAA	CCACTACACG
1390	1400	1410			
CAGAAGAGCC	TCTCCCTGTC	TCCGGGTAAA	TGA		

<125N26F6AA heavy chain amino acid sequence> (SEQ ID NO: 83)

10	20	30	40	50	60
MEFGLSWVFL	VALLRGVQCQ	VQLVESGGGV	VQPGRSLRLS	CAASGFTFSH	YGMHWVRQAP
70	80	90	100	110	120
GKGLEWVALI	WYDGSNKYYA	DSVKGRFTIS	RDNSKNTLYL	QMNSLRAEDT	AVYYCARDPL
130	140	150	160	170	180
AAGTSYFDYW	GQGTLVTVSS	ASTKGPSVFP	LAPSSKSTSG	GTAALGCLVK	DYFPEPVTVS
190	200	210	220	230	240
WNSGALTSGV	HTFPAVLQSS	GLYSLSSVVT	VPSSSLGTQT	YICNVNHKPS	NTKVDKKVEP
250	260	270	280	290	300
KSCDKTHTCP	PCPAPELLGG	PSVFLFPPKP	KDTLMISRTP	EVTCVVVDVS	HEDPEVKFNW

310	320	330	340	350	360
YVDGVEVHNA	KTKPREEQYN	STYRVVSVLT	VLHQDWLNGK	EYKCKVSNKA	LPAPIEKTIS
370	380	390	400	410	420
KAKGQPREPQ	VYTLPPSRDE	LTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTTPV
430	440	450	460	470	480
LDSDGSFFLY	SKLTVDKSRW	QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK	

<125N26F6AA light chain nucleic acid sequence> (SEQ ID NO: 84)

10	20	30	40	50	60
ATGTCGCCAT	CACAACTCAT	TGGGTTTCTG	CTGCTCTGGG	TTCCAGCCTC	CAGGGGTGAA
70	80	90	100	110	120
ATTGTGCTGA	CTCAGTCTCC	AGACTTTCAG	TCTGTGACTC	CAAAGGAGAA	AGTCACCATC
130	140	150	160	170	180
ACCTGCCGGG	CCAGTCAGAG	CATTGGTAGT	AGCTTACACT	GGTACCAGCA	GAAACCAGAT
190	200	210	220	230	240
CAGTCTCCAA	AGCTCCTCAT	CAAGTATGCT	TCCCAGTCCT	TCTCAGGGGT	CCCCTCGAGG
250	260	270	280	290	300
TTCAGTGGCA	GTGGATCTGG	GACAGATTTC	ACCCTCACCA	TCAATAGCCT	GGAAGCTGAA
310	320	330	340	350	360
GATGCTGCAG	CGTATTACTG	TCATCAGAGT	AGTAGTTTAC	CATTCACTTT	CGGCCCTGGG
370	380	390	400	410	420
ACCAAAGTGG	ATATCAAACG	AACTGTGGCT	GCACCATCTG	TCTTCATCTT	CCCGCCATCT
430	440	450	460	470	480
GATGAGCAGT	TGAAATCTGG	AACTGCCTCT	GTTGTGTGCC	TGCTGAATAA	CTTCTATCCC
490	500	510	520	530	540
AGAGAGGCCA	AAGTACAGTG	GAAGGTGGAT	AACGCCCTCC	AATCGGGTAA	CTCCCAGGAG
550	560	570	580	590	600
AGTGTCACAG	AGCAGGACAG	CAAGGACAGC	ACCTACAGCC	TCAGCAGCAC	CCTGACGCTG
610	620	630	640	650	660
AGCAAAGCAG	ACTACGAGAA	ACACAAAGTC	TACGCCTGCG	AAGTCACCCA	TCAGGGCCTG

670 680 690 700
AGCTCGCCCG TCACAAAGAG CTTCAACAGG GGAGAGTGTT AG
<125N26F6AA light chain amino acid sequence> (SEQ ID NO: 85)

10 20 30 40 50 60
MSPSQLIGFL LLWVPASRGE IVLTQSPDFQ SVTPKEKVTI TCRASQSIGS SLHWYQQKPD

70 80 90 100 110 120
QSPKLLIKYA SQSFSGVPSR FSGSGSGTDF TLTINSLEAE DAAAYYCHQS SSLPFTFGPG

130 140 150 160 170 180
TKVDIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE

190 200 210 220 230
SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC

DNAs encoding the entire 125M10AA antibody heavy chain region and the entire light chain region and the amino acid sequences of the entire heavy chain region and the entire light chain region are each shown below.

<125M10AA heavy chain nucleic acid sequence> (SEQ ID NO: 86)

10 20 30 40 50 60
ATGGATCTCA TGTGCAAGAA AATGAAGCAC CTGTGGTTCT TCCTCCTGCT GGTGGCGGCT

70 80 90 100 110 120
CCCAGATGGG TCCTGTCCCA GCTGCAGGTG CAGGAGTCGG GCCCAGGACT GGTGAAGCCT

130 140 150 160 170 180
TCGGAGACCC TGTCCCTCAT CTGCACTGTC TCTGGTGGCT CCATCAGGAC CAGTGGTTAC

190 200 210 220 230 240
TACTGGGGCT GGTTCGCCA GCCCCCAGGG AAGGGACTGG AGTGGATTGG GACTAGTCAT

250 260 270 280 290 300
AATAGTGGGA GCACCTACTA CAACCCGTCC CTCAAGAGTC GAGTCACCAT ATCCGTAGAC

310 320 330 340 350 360
ACGTCCAAGA ACCAGTTCTC CCTGAAGCTG AACTCTGTGA CCGCCGAGA CACGGCTGTG

370	380	390	400	410	420
TATTACTGTG	CGAGACAAGG	TTACGATTTT	AAAGTCAATA	TAGACGTCTG	GGGACAAGGG
430	440	450	460	470	480
ACCACGGTCA	CCGTCTCCTC	AGCCTCCACC	AAGGGCCCAT	CGGTCTTCCC	CCTGGCACCC
490	500	510	520	530	540
TCCTCCAAGA	GCACCTCTGG	GGGCACAGCG	GCCCTGGGCT	GCCTGGTCAA	GGACTACTTC
550	560	570	580	590	600
CCCGAACCGG	TGACGGTGTC	GTGGA ACTCA	GGCGCCCTGA	CCAGCGGCGT	GCACACCTTC
610	620	630	640	650	660
CCGGCTGTCC	TACAGTCCTC	AGGACTCTAC	TCCCTCAGCA	GCGTGGTGAC	CGTGCCCTCC
670	680	690	700	710	720
AGCAGCTTGG	GCACCCAGAC	CTACATCTGC	AACGTGAATC	ACAAGCCCAG	CAACACCAAG
730	740	750	760	770	780
GTGGACAAGA	AAGTTGAGCC	CAAATCTTGT	GACAAA ACTC	ACACATGCCC	ACCGTGCCCA
790	800	810	820	830	840
GCACCTGAAC	TCCTGGGGGG	ACCGTCAGTC	TTCCTCTTCC	CCCCAAAACC	CAAGGACACC
850	860	870	880	890	900
CTCATGATCT	CCCGGACCCC	TGAGGTCACA	TGCGTGGTGG	TGGACGTGAG	CCACGAAGAC
910	920	930	940	950	960
CCTGAGGTCA	AGTTCAACTG	GTACGTGGAC	GGCGTGGAGG	TGCATAATGC	CAAGACAAAG
970	980	990	1000	1010	1020
CCGCGGGAGG	AGCAGTACAA	CAGCACGTAC	CGTGTGGTCA	GCGTCCTCAC	CGTCCTGCAC
1030	1040	1050	1060	1070	1080
CAGGACTGGC	TGAATGGCAA	GGAGTACAAG	TGCAAGGTCT	CCAACAAAGC	CCTCCCAGCC
1090	1100	1110	1120	1130	1140
CCCATCGAGA	AAACCATCTC	CAAAGCCAAA	GGGCAGCCCC	GAGAACCACA	GGTGTACACC
1150	1160	1170	1180	1190	1200
CTGCCCCCAT	CCCGGGATGA	GCTGACCAAG	AACCAGGTCA	GCCTGACCTG	CCTGGTCAAA
1210	1220	1230	1240	1250	1260

GGCTTCTATC CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
 1270 1280 1290 1300 1310 1320
 TACAAGACCA CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
 1330 1340 1350 1360 1370 1380
 ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG
 1390 1400 1410 1420 1430
 GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA ATGA

<125M10AA heavy chain amino acid sequence> (SEQ ID NO: 87)

10 20 30 40 50 60
 MDLMCKMKH LWFFLLLVAA PRWVLSQLQV QESGPGLVKP SETLSLICTV SGGSIRTSGY
 70 80 90 100 110 120
 YWGWFRQPPG KGLEWIGTSH NSGSTYYNPS LKSRVTISVD TSKNQFSLKL NSVTAADTAV
 130 140 150 160 170 180
 YYCARQGYDF KVNIDVWGQG TTVTVSSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF
 190 200 210 220 230 240
 PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHNKPSNTK
 250 260 270 280 290 300
 VDKKVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED
 310 320 330 340 350 360
 PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA
 370 380 390 400 410 420
 PIEKTISKAK GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN
 430 440 450 460 470 480
 YKTPPVLDSDGSFFLYSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS LSLSPGK

<125M10AA light chain nucleic acid sequence> (SEQ ID NO: 88)

10 20 30 40 50 60
 ATGGAAGCCC CAGCTCAGCT TCTCTTCCTC CTGCTACTCT GGCTCCCAGA TACCACCGGA
 70 80 90 100 110 120
 GAAATTGTGT TGACACAGTC TCCAGCCACC CTGTCTTTGT CTCCAGGGGA AAGAGCCACC

130	140	150	160	170	180
CTCTCCTGCA	GGGCCAGTCA	GAGTGTTAGC	AGCTACTTAG	CCTGGTACCA	ACAGAAACCT
190	200	210	220	230	240
GGCCAGGCTC	CCAGGCTCCT	CATCTATGAT	GCATCCAACA	GGGCCACTGG	CATCCCAGCC
250	260	270	280	290	300
AGGTTTCAGTG	GCAGTGGGTC	TGGGACAGAC	TTCACTCTCA	CCATCAGCAG	CCTAGAGCCT
310	320	330	340	350	360
GAAGATTTTG	CAGTTTATTA	CTGTCAGCAG	CGTAGCAACT	GGCCGCTCAC	TTTCGGCGGA
370	380	390	400	410	420
GGGACCAAGG	TGGAGATCAA	ACGAACTGTG	GCTGCACCAT	CTGTCTTCAT	CTTCCCGCCA
430	440	450	460	470	480
TCTGATGAGC	AGTTGAAATC	TGGAAGTGCC	TCTGTTGTGT	GCCTGCTGAA	TAACTTCTAT
490	500	510	520	530	540
CCCAGAGAGG	CCAAAGTACA	GTGGAAGGTG	GATAACGCCC	TCCAATCGGG	TAACTCCCAG
550	560	570	580	590	600
GAGAGTGTCA	CAGAGCAGGA	CAGCAAGGAC	AGCACCTACA	GCCTCAGCAG	CACCCTGACG
610	620	630	640	650	660
CTGAGCAAAG	CAGACTACGA	GAAACACAAA	GTCTACGCCT	GCGAAGTCAC	CCATCAGGGC
670	680	690	700		
CTGAGCTCGC	CCGTCACAAA	GAGCTTCAAC	AGGGGAGAGT	GTTAG	

<125M10AA light chain amino acid sequence> (SEQ ID NO: 89)

10	20	30	40	50	60
MEAPAQLLFL	LLLWLPDTTG	EIVLTQSPAT	LSLSPGERAT	LSCRASQSVS	SYLAWYQQKP
70	80	90	100	110	120
GQAPRLLIYD	ASNRATGIPA	RFSGSGSGTD	FTLTISSLEP	EDFAVYYCQQ	RSNWPLTFGG
130	140	150	160	170	180
GTKVEIKRTV	AAPSVFIFPP	SDEQLKSGTA	SVVCLLNNFY	PREAKVQWKV	DNALQSGNSQ
190	200	210	220	230	
ESVTEQDSKD	STYSLSSTLT	LSKADYEKHK	VYACEVTHQG	LSSPVTKSFN	RGEC

Example 15 Construction of vectors for expression of recombinant antibodies

Vectors for expression of 263A17, 125M10AA, 125M165DAAA, 125N26F6AA, and 125Q54AAAA recombinant antibodies were constructed.

For the 263A17, 125M165DAAA, or 125N26F6AA antibody, a plasmid DNA containing the HV chain of each type of obtained antibody was used as a template. Primers used herein were designed to add restriction enzyme sites (*Sal* I on the 5' terminus and *Nhe* I on the 3' terminus) to the termini for ligation. Specifically, the primers used herein are as follows.

263A17:

5' primer for HV chain: A33 2-6A2 H VH3-23 *Sal* I (SEQ ID NO: 54)

5'-GCG ACT AAG TCG ACC ATG GAG TTT GGG CTG AGC TG-3'

3' primer for HV chain: A33 2-6A2 H VH3-23 *Nhe* I (SEQ ID NO: 55)

5'-TGG GCC CTT GGT GCT AGC TGA GGA GAC GGT GAC CG-3'

125M165DAAA:

5' primer for HV chain: M165H5*Sal* (SEQ ID NO: 56)

5'-AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGG TTT-3'

3' primer for HV chain: M165H3*Nhe* (SEQ ID NO: 57)

5'-AGA GAG AGA GGC TAG CTG AGG AGA CGG TGA CCA GGG TGC-3'

125N26F6AA:

5' primer for HV chain: N26H5*Sal*1 (SEQ ID NO: 58)

5'-AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGG TTT-3'

3' primer for HV chain: N26H3*Nhe*1 (SEQ ID NO: 59)

5'-AGA GAG AGA GGC TAG CTG AGG AGA CGG TGA CCA GGG TTC CC-3'

The HV of each type of A33 antibody was amplified by PCR (94°C for 3 minutes → 94°C for 10 seconds and 68°C for 45 seconds (35 cycles) → 72°C for 7 minutes). The amplified DNA fragment was digested with *Sal* I and *Nhe* I. The digested fragment was then introduced into an N5KG1-Val Lark vector (IDEC Pharmaceuticals, a modified vector of N5KG1 (US patent 6001358)) that had been

cleaved with the same enzymes. It was confirmed by sequencing with the use of the vector as a template that the inserted sequence was identical to the nucleotide sequence of the subcloned HV as determined by DNA nucleotide sequence analysis.

Subsequently, LV was inserted into the thus obtained plasmid vector into which the HV had been inserted. A plasmid DNA containing the LV chain of each type of obtained antibody was used as a template. Primers used herein were designed to add restriction enzyme sites (*Bgl* II on the 5' terminus and *Bsi*W I on the 3' terminus) to the termini for ligation. Specifically, primers used herein are as follows.

263A17:

5' primer for LV chain: A33 2-6A2 K L19 *Bgl* II (SEQ ID NO: 60)

5'-ATC ACA GAT CTC TCA CCA TGG ACA TGA GGG TCC CC-3'

3' primer for LV chain: A33 2-6A2 K L19 *Bsi*W I (SEQ ID NO: 61)

5'-ACA GAT GGT GCA GCC ACC GTA CGT TTA ATC TCC AG-3'

125M165DAAA:

5' primer for LV chain: M165K5L6*Bgl*II (SEQ ID NO: 62)

5'-AGA GAG AGA GAG ATC TCA CCA TGG AAG CCC CAG CTC AGC TTC TCT-3'

3' primer for LV chain: M165K3L6*Bsi*W I (SEQ ID NO: 63)

5'-AGA GAG AGA GCG TAC GTT TGA TTT CCA CCT TGG TCC CTT GGC-3'

125N26F6AA:

5' primer for LV chain: N26KA10 Minor L *Bgl* (SEQ ID NO: 64)

5'-AGA GAG AGA GAT CTC TCA CCA TGT CGC CAT CAC AAC TCA TTG GG-3'

3' primer for LV chain: N26KA10 Minor L *Bsi* (SEQ ID NO: 65)

5'-AGA GAG AGA GCG TAC GTT TGA TAT CCA CTT TGG TCC CAG GG-3',

The LV of each type of A33 antibody was amplified by PCR (94°C for 3 minutes → 94°C for 10 seconds and 68°C for 45 seconds (35 cycles) → 72°C for 7 minutes). The amplified DNA fragment was digested with *Bgl* II and *Bsi*W I. The digested fragment was then introduced into an N5KG1-HV vector that had been cleaved with the same enzymes. It was confirmed by sequencing with the use of the vector as a template that the inserted sequence was identical to the nucleotide sequence of the subcloned LV

as determined by DNA nucleotide sequence analysis.

For the 125M10AA or 125Q54AAAA antibody, a plasmid DNA containing the LV chain of each type of obtained antibody was used as a template. Primers used herein were designed to add restriction enzyme sites (*Bgl* II on the 5' terminus and *Bsi*W I on the 3' terminus) to the termini for ligation. Specifically, primers used herein are as follows.

125M10AA:

5' primer for LV chain: M10KBgl (SEQ ID NO: 66)

5'-AGAGAGAGAGAGATCTCACCATGGAAGCCCCAGCTCAGCTTCTCT-3'

3' primer for LV chain: M10KBsi (SEQ ID NO: 67)

5'-AGAGAGAGAGAGCGTACGTTTGATCTCCACCTTGGTCCCCTCCG-3'

125Q54AAAA:

5' primer for LV chain: Q54K5Bgl (SEQ ID NO: 68)

5'-AGAGAGAGAGAGATCTCACCATGGACATGAGGGTCCTCGCTCAGC-3'

3' primer for LV chain: Q54K3Bsi (SEQ ID NO: 69)

5'-AGAGAGAGAGAGCGTACGTTTGATCTCCAGCTTGGTCCCCTGG-3'

The LV of each type of A33 antibody was amplified by PCR (94°C for 3 minutes → 94°C for 10 seconds and 68°C for 45 seconds (35 cycles) → 72°C for 7 minutes). The amplified DNA fragment was digested with *Bgl* II and *Bsi*W I. The digested fragment was then introduced into a N5KG1-Val Lark vector that had been cleaved with the same enzymes. It was confirmed by sequencing with the use of the vector as a template that the inserted sequence was identical to the nucleotide sequence of the subcloned LV as determined by DNA nucleotide sequence analysis.

Subsequently, HV was inserted into the thus obtained plasmid vector into which the LV had been inserted. A plasmid DNA containing the HV chain of each type of obtained antibody was used as a template. Primers used herein were designed to add restriction enzyme sites (*Sal* I on the 5' terminus and *Nhe* I on the 3' terminus) to the termini for ligation. Specifically, primers used herein are as follows.

125 M10AA:

5' primer for HV chain: M10H5Sal (SEQ ID NO: 70)

5'-AGA GAG AGA GGT CGA CCA CCA TGG ATC TCA TGT GCA AGA AAA TGA AGC-3'

3' primer for HV chain: M10H3Nhe (SEQ ID NO: 71)

5'-AGA GAG AGA GGC TAG CTG AGG AGA CGG TGA CCG TGG TCC CT-3'

125 Q54AAAA:

5' primer for HV chain: Q54H5Sal (SEQ ID NO: 72)

5'-AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGC TTT-3'

3' primer for HV chain: Q54H3Nhe (SEQ ID NO: 73)

5'-AGA GAG AGA GGC TAG CTG AGG AGA CGG TGA CCA GGG TTC CC-3'

The HV of each type of A33 antibody was amplified by PCR (94°C for 3 minutes → 94°C for 10 seconds and 68°C for 45 seconds (35 cycles) → 72°C for 7 minutes). The amplified DNA fragment was digested with *Sal* I and *Nhe* I. The digested fragment was then introduced into an N5KG1-LV vector that had been cleaved with the same enzymes. It was confirmed by sequencing with the use of the vector as a template that the inserted sequence was identical to the nucleotide sequence of the subcloned HV as determined by DNA nucleotide sequence analysis.

Table 5 lists the nucleotide sequences of the synthetic DNAs. Table 6 lists the names of the recombinant vectors and the names of the antibodies produced.

Table 5

No	Primer name	Sequence (5' to 3')	Length	SEQ ID NO:
1	GPAHVR3Nhe	GCC CTT GGT GCT AGC TGA AGA GAC GGT GAC CAG AGT CCC TTG	42	1
2	GPALVR3Bsi	GTG CAC GCC GCT GGT CAG GGC GCC TG	26	2
3	M13FW	GTA AAA CGA CGG CCA GTG	18	3
4	M13RV	CAG GAA ACA GCT ATG AC	17	4
5	GPAHV2F5Sal	AGA GAG AGG TCG ACC CAC CAT GAA CTT TGG GCT GAG CTT AGT T	43	5
6	GPALV2FBgl	AGA GAG AGA GAT CTC TCA CCA TGG GCA TCA AGA TGG AGT TTC AG	44	6
7	A33-F2	GCA GAC GAA TTC AAG ACC ATG GTG GGG AAG AT	32	13
8	A33-R1	CTC GAG CGG CCG CTC TGC TGC TGG CCT GTC ACT GGT CGA GGT G	43	14
9	GPA-EXCRR2	CTC GAG CGG CCG CCA GTT CAT GGA GGG AGA TCT GAC G	37	15
10	IgG1p	TCT TGT CCA CCT TGG TGT TGC TGG GCT TGT G	31	16
11	hk-2	GTT GAA GCT CTT TGT GAC GGG CGA GC	26	17
12	IgG2p/G134	TGC ACG CCG CTG GTC AGG GCG CCT GAG TTC C	31	18
13	hk-5	AGG CAC ACA ACA GAG GCA GTT CCA GAT TTC	30	19
14	hh-2	GCT GGA GGG CAC GGT CAC CAC GCT G	25	20
15	hh-4	GGT GCC AGG GGG AAG ACC GAT GG	23	21
16	A33 2-6A2 H VH3-23 Sail	GCG ACT AAG TCG ACC ATG GAG TTT GGG CTG AGC TG	35	54
17	A33 2-6A2 H VH3-23 NheI	TGG GCC CTT GGT GCT AGC TGA GGA GAC GGT GAC CG	35	55
18	M165H5Sal	AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGG TTT	45	56
19	M165H3Nhe	AGA GAG AGA GGC TAG CTG AGG AGA CCG TGA CCA GGG TGC	39	57
20	N26H5Sal1	AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGG TTT	45	58
21	N26H3Nhe1	AGA GAG AGA GGC TAG CTG AGG AGA CCG TGA CCA GGG TTC CC	41	59
22	A33 2-6A2 K L19 BglII	ATC ACA GAT CTC TCA CCA TGG ACA TGA GGG TCC CC	35	60
23	A33 2-6A2 K L19 BsiWI	ACA GAT GGT GCA GCC ACC GTA CGT TTA ATC TCC AG	35	61
24	M165K5L6Bgl2	AGA GAG AGA GAG ATC TCA CCA TGG AAG CCC CAG CTC AGC TTC TCT	45	62
25	M165K3L6BsiW1	AGA GAG AGA GCG TAC GTT TGA TTT CCA CCT TGG TCC CTT GGC	42	63
26	N26KA10Minor L Bgl	AGA GAG AGA GAT CTC TCA CCA TGT CGC CAT CAC AAC TCA TTG GG	44	64
27	N26KA10Minor L Bsi	AGA GAG AGA GCG TAC GTT TGA TAT CCA CTT TGG TCC CAG GG	41	65
28	M10KBgl	AGA GAG AGA GAG ATC TCA CCA TGG AAG CCC CAG CTC AGC TTC TCT	45	66
29	M10KBSi	AGA GAG AGA GCG TAC GTT TGA TCT CCA CCT TGG TCC CTC CG	41	67
30	Q54K5Bgl	AGA GAG AGA GAG ATC TCA CCA TGG ACA TGA GGG TCC TCG CTC AGC	45	68
31	Q54K3Bsi	AGA GAG AGA GCG TAC GTT TGA TCT CCA GCT TGG TCC CCT GG	41	69
32	M10H5Sal	AGA GAG AGA GGT CGA CCA CCA TGG ATC TCA TGT GCA AGA AAA TGA AGC	48	70
33	M10H3Nhe	AGA GAG AGA GGC TAG CTG AGG AGA CCG TGA CCG TGG TCC CT	41	71
34	Q54H5Sal	AGA GAG AGA GGT CGA CCA CCA TGG AGT TTG GGC TGA GCT GGC TTT	45	72
35	Q54H3Nhe	CGG GGT ACG TGC CAA GCA TCC TCG TG	41	73
36	H_3UTR1848	ATG CTG GGC GCC CGG GAA GTA TGT AC	26	74
37	H_3UTR1875	GAA AGA TGA GCT GGA GGA CCG CAA TA	26	75
38	L_3UTR 823	CCA AGG GCC CAT CCG TCT TCC CCC TGG CAC	30	76
39	hh-1	GAC ACC CTC ATG ATC TCC CGG ACC	24	77
40	CMVH903F	TGT TCT CCG GCT GCC CAT TGC TCT	24	78
41	CMVHR1303	GGT CCG GGA GAT CAT GAG GGT GTC CTT	27	79
42	hh-6	TGG CTG CAC CAT CTG TCT TCA TCT TC	26	80
43	hk-1		26	81

Table 6

Antibody	Vector name	Subclass	Recombinant antibody name
263A17	N5KG1-Val Lark	IgG1	rec263
125M10AA	N5KG1-Val Lark	IgG1	recM10
125M165DAAA	N5KG1-Val Lark	IgG1	recM165
125N26F6AA	N5KG1-Val Lark	IgG1	recN26
125Q54AAAA	N5KG1-Val Lark	IgG1	recQ54

Example 16 Preparation of recombinant antibodies

Host cells were transfected with the vectors for expression of recombinant antibodies constructed in Example 15, thereby preparing recombinant-antibody-expressing cells. Host cells to be used for expression were CHO cells of a dhfr-deficient cell line (ATCC CRL-9096), CHO-Ras (Katakura Y., et al., Cytotechnology, 31: 103-109, 1999), or HEK293T (ATCC CRL-11268), for example.

Host cells were transfected with each type of vector by electroporation, lipofection, or the like. Approximately 2 μ g of the antibody expression vector was linearized using restriction enzymes and then subjected to electroporation using a Bio-Rad electrophoreter under conditions of 350 V and 500 μ F. 4×10^6 CHO cells were thus transfected with each type of gene, and then the cells were inoculated on a 96-well culture plate. Lipofection was performed using LipofectAMINE Plus (produced by Gibco BRL) according to the manual included therein. After transfection with such vector, a drug corresponding to the selection marker used in the expression vector was added, and then culture was continued. After confirmation of colonies, antibody-expressing cell lines were selected by the method described in Example 6. Antibody purification was performed from the selected cells by washing twice with PBS after adsorption and then eluting using 20 mM (glycine) sodium citrate and a 50 mM NaCl (pH 2.7) buffer using a Mab Select Protein A 3.2×10 cm column (produced by Amersham Pharmacia Biotech). The eluted solution was neutralized with a 50 mM

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sodium phosphate buffer (pH 7.0). Next, purification was performed using a Hitrap Q HP Sepharose^{*} column (produced by Amersham Pharmacia Biotech), which is an anion exchange column, and then similarly performed using a Hitrap SP HP Sepharose column (produced by Amersham Pharmacia Biotech) which is a cation column. The thus prepared antibody solution was substituted with PBS using a dialysis membrane (10000 cut-off produced by Spectrum Laboratories) and then sterilized by filtration using a membrane filter MILLEX-GV (produced by Millipore) with a pore size of 0.22 μ m. Thus purified antibodies with purity of at least 95% or higher and with endotoxin levels of 0.1 EU/mg or less endotoxin were obtained. The concentration of each type of recombinant purified anti-A33 antibody was obtained by measurement of absorbance at 280 nm and calculation with 1.4 OD being equivalent to 1 mg/mL (antibody concentration).

Example 17 Test of the reactivity of recombinant antibodies

The reactivity of each type of recombinant antibody obtained in Example 16 to A33-antigen-expressing human colorectal cancer cell line COLO205 cells, LS174T cells (ATCC No. CL-188), or NCI-H508 cells (ATCC No. CCL-253) was examined by FCM. The reactivity of human colorectal cancer cell line HT-29 cells (ATCC No. HTB-38) expressing no A33 antigen was also examined as negative control cells. The test method conducted herein was similar to that in Example 9.

Table 7 shows the results. For COLO205 cells, the half value of the mean fluorescence intensity was determined to be 45. For LS174T cells, the half value of the mean fluorescence intensity was determined to be 100. For NCI-H508 cells, the half value of the mean fluorescence intensity was determined to be 175. When an antibody concentration required to reach such value was $10 \leq x < 100$ ng/ml, the reactivity was represented by +++, when the same was $100 \leq x < 1000$ ng/ml, the reactivity was represented by ++, and when the same was $1000 \leq x < 10000$ ng/ml, the reactivity was represented by +. When no binding was observed, the reactivity was represented by -. Each type of recombinant antibody showed binding to all cells expressing the A33

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antigen.

Table 7

Antibody name	COLO205	LS174T	NCI-H508	HT-29
Anti-DNP-IgG1	-	-	-	-
cA33	+++	+++	+++	-
rec263	++	++	++	-
recM10	+++	+++	+++	-
recM165	++	++	++	-
recN26	++	+++	+++	-
recQ54	+++	+++	+++	-

Half value of the mean fluorescence intensity 45	Half value of the mean fluorescence intensity 100	Half value of the mean fluorescence intensity 175
10<=x<100 ng/ml; +++	10<=x<100 ng/ml; +++	10<=x<100 ng/ml; +++
100<=x<1000 ng/ml; ++	100<=x<1000 ng/ml; ++	100<=x<1000 ng/ml; ++
1000<=x<10000 ng/ml; +	1000<=x<10000 ng/ml; +	1000<=x<10000 ng/ml; +
No binding; -	No binding; -	No binding; -

Example 18 Competition test regarding each type of recombinant antibody and mouse anti-A33 antibody

Whether or not each type of type of recombinant antibody obtained in Example 16 recognizes an epitope similar to that of the mouse anti-A33 antibody was examined by a competition test using FCM. The test method conducted herein was similar to that in Example 10.

Similar to the results for each type of purified monoclonal antibody in Example 10, rec263 and recM10 were classified as “non-blockers,” recM165 and recN26 were classified as “blockers,” and recQ54 was classified as “a partial blocker.” Table 8 shows the results.

Table 8

Antibody name	Inhibition (%)	Classification
cA33	94.6	Blocker
rec263	15.3	Non-blocker
recM10	0.2	Non-blocker
recM165	93.4	Blocker
recN26	95.3	Blocker
recQ54	44.6	Partial blocker

Example 19 Test of the cytotoxicity of recombinant antibodies

ADCC and CDC of the recombinant antibodies obtained in Example 16 were determined. In the ADCC assay, 5,000 ⁵¹Cr-labeled target cells (COLO205 or NCI-H508) and 500,000 healthy human peripheral blood mononuclear leukocytes obtained by the method described in Example 11 were cultured in a V-bottom 96-well plate (produced by Coaster) at a total volume of 200 μ L with antibody having each concentration at 37°C in the presence of 5% CO₂ for 4 hours.

In the CDC assay, 5000 ⁵¹Cr-labeled target cells (COLO205 or NCI-H508) and human serum-derived complements (produced by Sigma) at a final concentration of 5% were cultured in a V-bottom 96-well plate at a total volume of 200 μ L with antibody

having each concentration at 37°C in the presence of 5% CO₂ for 4 hours.

The test method conducted herein was similar to that in Example 12.

Fig. 2A to Fig. 2D and Table 9 show the results. In the case of ADCC against COLO205 cells as target cells, the half value of the specific lysis (%) was determined to be 12.5%. In the case of ADCC against NCI-H508 cells as target cells, the half value of the specific lysis (%) was determined to be 30%. When an antibody concentration required to reach such value was $1 \leq x < 10$ ng/ml, the cytotoxicity was represented by +++, when the same was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by ++, when the same was $100 \leq x < 1000$ ng/ml, the cytotoxicity was represented by +, and when no specific lysis (%) was obtained, the cytotoxicity was represented by -. In the case of CDC against COLO205 cells as target cells, the half value of the specific lysis (%) was determined to be 7%. In the case of CDC against NCI-H508 cells as target cells, the half value of the specific lysis (%) was determined to be 20%. When an antibody concentration required to reach such value was $10 \leq x < 100$ ng/ml, the cytotoxicity was represented by +++, when the same was $100 \leq x < 1000$ ng/ml, the cytotoxicity was represented by ++, when the same was $x \geq 1000$ ng/ml, the cytotoxicity was represented by +, and when no specific lysis (%) was observed, the cytotoxicity was represented by -. In the case of ADCC, cA33, recM10, and recQ54 showed high cytotoxicity, while in the case of CDC, recM10 showed high cytotoxicity.

Table 9

Cell name	COLO205			NCI-H508	
Antibody name	ADCC	CDC		ADCC	CDC
Anti-DNP-IgG1	-	-		-	-
cA33	+++	±		++	++
rec263	++	++		++	++
recM10	+++	+++		++	+++
recM165	++	+		++	++
recN26	++	+		++	++
recQ54	+++	++		++	++

Half value of specific lysis (%) 12.5% Half value of specific lysis (%) 7% Half value of specific lysis (%) 30% Half value of specific lysis (%) 20%

1<=x<10 ng/ml; +++ 10<=x<100 ng/ml; +++ 1<=x<10 ng/ml; +++ 10<=x<100 ng/ml; +++

10<=x<100 ng/ml; ++ 100<=x<1000 ng/ml; ++ 10<=x<100 ng/ml; ++ 100<=x<1000 ng/ml; ++

100<=x<1000 ng/ml; + 1000<=x<10000 ng/ml; + 100<=x<1000 ng/ml; + 1000<=x<10000 ng/ml; +

No specific lysis; - x>=10000 ng/ml;± No specific lysis; - x>=10000 ng/ml;±

No specific lysis; - No specific lysis; - No specific lysis; - No specific lysis; -

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Example 20 Western blot analysis of the purified antibodies and recombinant antibodies

Mouse anti-A33 and humanized A33 antibodies have been reported to recognize conformational epitopes. Specifically, in the cases of these antibodies, it has been reported that no reactivity was observed via Western blot analysis under reducing conditions (5% β -mercaptoethanol). Hence, Western blot analysis was performed in order to examine the reactivity of the purified human anti-A33 and recombinant antibodies.

The shA33EX-hFc protein prepared in Example 3 was separated by SDS-PAGE using 10% to 20% polyacrylamide gradient gel (produced by Daiichi Pure Chemicals) under reducing (5% β -mercaptoethanol) and non-reducing conditions. At this time, the shA33EX-hFc protein was diluted to 2.5 ng per lane. In the meantime, biotinylated SDS-PAGE Standard BroadRange (produced by Bio-Rad Laboratories) was also applied to one lane as a marker. The shA33EX-hFc protein was blotted onto a PVDF membrane using Panther Semidry Electroblotter (Daiichi Pure Chemicals) at 150 mA/membrane for 1 hour. The membrane onto which the protein had been blotted was washed using a TBS buffer and TBS (TTBS) containing 0.05% Tween. Blocking was performed using BlockAce* (produced by Dainippon Pharmaceutical). Washing was performed twice with TTBS. Each type (1 μ g/ml) of antibody purified from 125M10AA, 125Q54AAAA, 125M96ABA, 125Q47BA, and 125R5AAAA hybridomas was caused to react at room temperature for 60 minutes. In the meantime, each type (1 μ g/ml) of recombinant antibody including the chimeric anti-A33 antibody, 125M165DAAA (that is, recM165), and 125N26F6AA (that is, recN26), was caused to react at room temperature for 60 minutes. After washing with TTBS, a goat anti-human Kappa chain F(ab')₂ antibody (produced by Biosource) labeled with horseradish peroxidase (diluted 1000-fold) was used as an antibody for detection. At this time, streptavidin labeled with horseradish peroxidase (diluted 3000 fold) was also added and caused to react so as to detect markers. After washing twice with TTBS and then once with PBS, band detection was performed using Western blotting detection system ECL-plus (produced by Amersham Biosciences). Chemoluminescence was

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incorporated using an image analyzer LAS-100 (produced by Fuji Photo Film) and then image processing was performed.

Fig. 3A and Fig. 3B show the results. It was found as a result that only 125Q54AAAA reacted with a protein band of approximately 67 kD even under reducing conditions. In the cases of the other antibodies, reaction was observed only under non-reducing conditions, similar to the case of the chimeric anti-A33 antibody.

Example 21 Immunohistochemical analysis of the purified antibodies and recombinant antibodies

To evaluate whether or not each type of human anti-A33 antibody is equivalent to the chimeric anti-A33 antibody in terms of specificity and selectivity, the reactivity of tumor tissue sections and that of normal tissue sections were analyzed by immunohistochemical analysis.

(1) Fluorescent labeling of the purified antibodies and recombinant antibodies

Each type of purified monoclonal antibody prepared in Example 8 was directly labeled with Alexa FluorTM 488 (produced by Molecular Probe). Each type of recombinant antibody (rec263, 125M10AA, recM165, recN26, and 125Q54AAA) prepared in Example 16 was also directly labeled with the same. Similarly, the chimeric anti-A33 antibody was directly labeled as a positive control and an anti-DNP-IgG1 antibody was directly labeled as a negative control. Fluorescent labeling was performed for the purified and recombinant antibodies as described below. Alexa FluorTM 488 was bound to each type of anti-A33 antibody prepared in Example 8 or 16 according to the instructions included therein. 50 µl of a 1 M carbonate buffer was added to 2 mg/ml of each type of purified antibody and to 0.5 ml of each type of recombinant antibody. The solutions were mixed with Alexa FluorTM 488, followed by 1 hour of reaction at room temperature while stirring of the solutions took place. Hydroxylamine was added to stop the reactions. Each of the mixed solutions was applied to a gel filtration column (NAP5, produced by Amersham Pharmacia Biotech), thereby removing Alexa FluorTM 488 that had not bound to the antibodies. 4 to 6

fluorescent substances bound to 1 molecule of antibody under these conditions. The fluorescent-labeled antibodies bound to COLO205 cells and showed binding activity equivalent to those of unlabeled antibodies.

(2) Immunohistochemistry

Tissue sections used herein were frozen human adult colon cancer tissue sections (produced by BioChain), frozen human adult normal colon tissue sections (produced by BioChain), frozen human adult small intestine tissue sections (produced by BioChain), and frozen human adult stomach tissue sections (produced by BioChain). Blocking was performed for 1 to 2 hours at room temperature using PBS containing 10% goat serum (produced by Gibco BRL). Washing was performed twice with PBS. Each type of purified monoclonal or recombinant antibody labeled with Alexa FluorTM488 in Example 21 (1) was caused to react at 1 µg/ml at room temperature for 30 to 60 minutes. Subsequently, the sections were mounted and then observed under a fluorescence microscope (BX51 produced by Olympus). The images were analyzed using DP70 (produced by Olympus).

Figs. 4 to 6 show the results. Similar to the results of immunohistostaining of colorectal cancer tissues as reported by Garin-Chesa P et al. (Int. J. Oncology 1996 9: 465-471), extensive, uniform, and strong staining of glandular epithelium cells or dysplastic gland structures of colon cancer tissues were observed in the cases of the chimeric anti-A33 antibody, rec263, 125M10AA, recM165, recN26, and 125Q54AAA (Fig. 4). Moreover, it was observed that normal small intestine tissues (Fig. 5) and normal colon tissues (Fig. 6) had been stained in a manner similar to that in the case of the chimeric anti-A33 antibody. In contrast, no staining of normal gastric tissues was observed even in the cases of the antibodies, similar to the results of the aforementioned paper. Furthermore, in the case of the anti-DNP-IgG1 antibody as a negative control, staining was not observed in any tissues.

Example 22 Effects of antibodies purified from hybridomas and recombinant antibodies on mouse tumor-bearing models

The effects of the human anti-A33 recombinant antibodies obtained in Example 16 were examined using mouse tumor-bearing models according to the method described below. The cells of colorectal cancer cell lines used herein were COLO205 cells and NCI-H508 cells.

A method for preparing a mouse tumor-bearing model using the COLO205 cell line is described below. The cells of the colorectal cancer cell line COLO205 were subcutaneously engrafted at 5×10^6 /mouse in the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan). On days 1, 2, 7, and 10 after the engrafting of the cells, the chimeric anti-A33 or rec263 antibody (dissolved in 200 μ l of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 10 μ g/mouse or 100 μ g/mouse to tumor-bearing mice (10 mice per group). Tumor sizes were measured on days 7, 9, 11, 14, 17, and 21 after the engrafting of the cells. A human anti-DNP-IgG1 antibody was used as a negative control antibody in amounts identical to those of the above antibodies. "Vehicle" denotes 1% nude mouse serum-containing PBS (200 μ l) that was used as medium for dissolution upon antibody administration.

A method for preparing a mouse tumor-bearing model using the NCI-H508 cell line is described below. The cells of the colorectal cancer cell line NCI-H508 and Matrigel (produced by Becton Dickinson Bioscience) comprising mouse malignant sarcoma (which improves the survival and proliferation of tumor cells) were subcutaneously engrafted at 1×10^7 /mouse in the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan), so that the ratio of NCI-H508 to Matrigel would be 1:1. The chimeric anti-A33 or rec263 antibody (dissolved in 200 μ l of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 10 μ g/mouse or 100 μ g/mouse to tumor-bearing mice (10 mice per group) on days 1, 4, and 7 after the engrafting of the cells. Tumor sizes were measured on days 7, 11, 14, 18, 21, 27, 33, 40, 48, 55, and 62 after the engrafting of the cells. A human anti-DNP-IgG1 antibody was used as a negative control antibody in amounts identical to those of the above antibodies. "Vehicle" denotes 0.1% nude mouse serum-containing PBS (200 μ l) that was used as a medium for dissolution upon antibody administration.

Fig. 7 shows the results of the above experiment.

Regarding the mouse line in which the COLO205 cell line had been engrafted, significant tumor suppression ($p < 0.05$) was observed on days 7, 9, and 11 after the engrafting of the cells in the case of the group to which the rec263 antibody had been administered at 10 $\mu\text{g}/\text{mouse}$, compared with the group to which the vehicle had been administered. Compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant differences in tumor size ($p < 0.05$) were observed on days 7, 9, 11, and 14 after the engrafting of the cells. Furthermore, compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed on days 14 and 17 after the engrafting of the cells in the case of the group to which the rec263 antibody had been administered at 100 $\mu\text{g}/\text{mouse}$. In contrast, significant tumor suppression ($p < 0.05$) was observed on days 7 and 11 after the engrafting of the cells in the case of the group to which the chimeric anti-A33 recombinant antibody had been administered at 10 $\mu\text{g}/\text{mouse}$, compared with the group to which the vehicle had been administered. Compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed on days 7, 9, 11, 14, 17, and 21 after the engrafting of the cells. Moreover, significant tumor suppression ($p < 0.05$) was observed on days 7, 9, and 14 after the engrafting of the cells in the case of the group to which the chimeric anti-A33 recombinant antibody had been administered at 100 $\mu\text{g}/\text{mouse}$, compared with the group to which the vehicle had been administered. Compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed on days 7, 9, 11, 14, 17, and 21 after the engrafting of the cells (Fig. 7A).

On the other hand, regarding the mouse line in which the NCI-H508 cell line had been engrafted, no antitumor effects were exerted in the case of the group to which the rec263 antibody had been administered at 10 $\mu\text{g}/\text{mouse}$. In contrast, significant tumor suppression ($p < 0.05$) was observed more than 18 days after the engrafting of the cells in the case of the group to which the rec263 antibody had been administered at 100

μg/mouse, compared with the group to which the vehicle had been administered. Moreover, compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed on nearly all measuring days. In contrast, significant tumor suppression ($p < 0.05$) was observed on days 21, 55, and 62 after the engrafting of the cells in the case of the group to which the chimeric anti-A33 recombinant antibody had been administered at 10 μg/mouse, compared with the group to which the vehicle had been administered. Furthermore, compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed on days 7, 21, and 33 after the engrafting of the cells. Moreover, significant tumor suppression ($p < 0.05$) was observed on nearly all measuring days in the case of the group to which the chimeric anti-A33 recombinant antibody had been administered at 100 μg/mouse, compared with the group to which the vehicle had been administered. Furthermore, compared with the group to which the anti-DNP-IgG1 antibody had been administered, significant tumor suppression ($p < 0.05$) was observed by day 33 after the engrafting of the cells.

As described above, it was demonstrated that the antibodies of the present invention had high antitumor effects on the mouse tumor-bearing models using 2 types of the colorectal cancer cell lines (Fig. 7B).

The effects of the antibodies purified from human anti-A33-producing hybridomas and the human anti-A33 recombinant antibodies were examined using the mouse tumor-bearing models. The cells of colorectal cancer cell lines used herein were COLO205 cells and NCI-H508 cells.

(Antitumor effects of the antibodies purified from 125M10AA, 125M165DAAA and 125M96ABA hybridomas on the COLO205 cell line)

The cells of the colorectal cancer cell line COLO205 were subcutaneously engrafted at 5×10^6 /mouse in the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan). On days 1, 3, 7, 10, 14, and 17 after the engrafting of the cells, the 125M10AA, 125M165DAAA, or 125M96ABA antibody (dissolved in 200

μ l of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 20 μ g/mouse to tumor-bearing mice (15 mice per group in the case of the group to which the vehicle was administered and 10 mice per group in the other cases). Tumor sizes were measured on days 7, 10, 12, 14, and 17 after the engrafting of the cells. "Vehicle" denotes 1% nude mouse serum-containing PBS (200 μ l) that was used as medium for dissolution upon antibody administration.

Fig. 7C shows the results of the above experiment. In Fig. 7C, M10 denotes the 125M10AA antibody, M96 denotes the 125M96ABA antibody, and M165 denotes 125M165DAAA antibody. Significant tumor suppression ($p < 0.05$) was observed on all measuring days after the engrafting of the cells in the case of the group to which the 125M10AA antibody had been administered, compared with the group to which the vehicle had been administered. Furthermore, significant tumor suppression ($p < 0.05$) was observed on days 12, 14, and 17 after the engrafting of the cells in the case of the group to which the 125M165DAAA antibody had been administered, compared with the group to which the vehicle had been administered. In contrast, significant tumor suppression ($p < 0.05$) was observed on days 12 and 14 after the engrafting of the cells in the case of the group to which the 125M96ABA antibody had been administered, compared with the group to which the vehicle had been administered. (Antitumor effects of N26 and M165 recombinant antibodies on COLO205 and NCI-H508 cell lines)

The cells of the colorectal cancer cell line COLO205 were subcutaneously engrafted at 5×10^6 /mouse in the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan). On days 1, 3, and 6 after the engrafting of the cells, the recN26 or recM165 antibody (dissolved in 200 μ l of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 10 μ g/mouse or 100 μ g/mouse to tumor-bearing mice (10 mice per group). Tumor sizes were measured on days 8, 10, 13, 15, 17, 20, and 23 after the engrafting of the cells.

Fig. 7D shows the results of the above experiment. In Fig. 7D, M165-10 denotes the recM165 antibody (administered at 10 μ g/mouse), M165-100 denotes the

recM165 antibody (administered at 100 µg/mouse), N26-10 denotes the recN26 antibody (administered at 10 µg/mouse), and N26-100 denotes the recN26 antibody (100 µg/mouse). Significant tumor suppression ($p < 0.05$) was observed on days 10 and 13 after the engrafting of the cells in the case of the group to which the recN26 antibody had been administered at 10 µg/head, compared with the group to which the vehicle had been administered. Furthermore, significant tumor suppression ($p < 0.05$) was observed on days 8, 10, 13, 15, 17, and 20 after the engrafting of the cells in the case of the group to which the recN26 antibody had been administered at 100 µg/head, compared with the group to which the vehicle had been administered. Furthermore, significant tumor suppression ($p < 0.05$) was observed on days 8, 10, 13, 15, 17, and 20 after the engrafting of the cells in the case of the group to which the recM165 antibody had been administered at 100 µg/head, compared with the group to which the vehicle had been administered.

The cells of the colorectal cancer cell line NCI-H508 and Matrigel (produced by Becton Dickinson Bioscience) comprising mouse malignant sarcoma (that improves the survival and proliferation of engrafted tumor cells) were subcutaneously engrafted at 1×10^7 /mouse to the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan), so that the ratio of NCI-H508 to Matrigel would be 1 : 1. The recN26 or recM165 antibody (dissolved in 200 µl of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 10 µg/mouse or 100 µg/mouse to tumor-bearing mice (12 mice per group in the case of the group to which the vehicle was administered and 10 mice per group in the other cases) on days 1, 4, and 7 after the engrafting of the cells. Tumor sizes were measured on days 11, 18, 28, 36, 43, 50, 57, and 64 after the engrafting of the cells.

Fig. 7E shows the results of the above experiment. In Fig. 7E, N26-10 denotes the recN26 antibody (administered at 10 µg/mouse), N26-100 denotes the recN26 antibody (administered at 100 µg/mouse), M165-10 denotes the recM165 antibody (administered at 10 µg/mouse), and M165-100 denotes the recM165 antibody (administered at 100 µg/mouse). Regarding the mouse line to which the NCI-H508 cell

line had been engrafted, significant tumor suppression ($p < 0.05$) was observed on days 11, 18, 36, and 43 after the engrafting of the cells in the case of the group to which the recN26 antibody had been administered at 10 $\mu\text{g}/\text{mouse}$, compared with the group to which the vehicle had been administered. Furthermore, compared with the group to which the vehicle had been administered, significant tumor suppression ($p < 0.05$) was observed on days 11, 18, 28, 36, and 50 after the engrafting of the cells in the case of the group to which the recN26 antibody had been administered at 100 $\mu\text{g}/\text{mouse}$. In contrast, significant tumor suppression ($p < 0.05$) was observed on days 11 and 18 after the engrafting of the cells in the case of the group to which the recM165 antibody had been administered at 10 $\mu\text{g}/\text{mouse}$, compared with the group to which the vehicle had been administered. Compared with the group to which the vehicle had been administered, significant tumor suppression ($p < 0.05$) was observed on all measuring days after the engrafting of the cells in the case of the group to which the recM165 antibody had been administered at 100 $\mu\text{g}/\text{mouse}$.

(Antitumor effects of the M10 and Q54 recombinant antibodies on the NCI-H508 cell line)

The cells of the colorectal cancer cell line NCI-H508 and Matrigel (produced by Becton Dickinson Bioscience) comprising mouse malignant sarcoma (that improves the survival and proliferation of engrafted tumor cells) were subcutaneously engrafted at $1 \times 10^7/\text{mouse}$ to the dorsal regions of 6-week-old Balb/c nude mice (purchased from CLEA Japan), so that the ratio of NCI-H508 to Matrigel would be 1 : 1. The recM10 or recQ54 antibody (dissolved in 200 μl of 1% nude mouse serum-containing PBS) was administered intraperitoneally at 10 $\mu\text{g}/\text{mouse}$ or 100 $\mu\text{g}/\text{mouse}$ to tumor-bearing mice (10 mice per group) on days 1, 4, and 7 after the engrafting of the cells. Tumor sizes were measured on days 14, 21, 28, 35, 42, 49, 56, and 63 after the engrafting of the cells.

Fig. 7F shows the results of the above experiment. In Fig. 7F, M10-10 denotes the recM10 antibody (administered at 10 $\mu\text{g}/\text{mouse}$), M10-100 denotes the recM10 antibody (administered at 100 $\mu\text{g}/\text{mouse}$), Q54-10 denotes the recQ54 antibody (administered at 10 $\mu\text{g}/\text{mouse}$), and Q54-100 denotes the recQ54 antibody (administered

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at 100 µg/mouse). Regarding the mouse line to which the NCI-H508 cell line had been engrafted, significant tumor suppression ($p < 0.05$) was observed on days 14, 21, 28, 42, 49, and 56 after the engrafting of the cells in the case of the group to which the recM10 antibody had been administered at 10 µg/mouse, compared with the group to which the vehicle had been administered. Furthermore, compared with the group to which the vehicle had been administered, significant tumor suppression ($p < 0.05$) was observed on days 14, 21, 28, 35, 42, 49, and 56 after the engrafting of the cells in the case of the group to which the recM10 antibody had been administered at 100 µg/mouse. In contrast, significant tumor suppression ($p < 0.05$) was observed on days 28 and 42 after the engrafting of the cells in the case of the group to which the recQ54 antibody had been administered at 10 µg/mouse, compared with the group to which the vehicle had been administered. Furthermore, compared with the group to which the vehicle had been administered, significant tumor suppression ($p < 0.05$) was observed on days 14, 21, 28, 35, 42, and 56 after the engrafting of the cells in the case of the group to which the recQ54 antibody had been administered at 100 µg/mouse.

Persons skilled in the art would easily understand that various modifications and changes of the present invention are feasible within the technical idea and the scope of the invention as disclosed in the attached claims. The present invention is intended to include such modifications and changes.

Industrial Applicability

According to the present invention, a preventive or therapeutic agent for diseases due to A33-expressing cells, and in particular, a molecule that is also useful as a malignant tumor remedy for patients with A33 polymorphism, are provided.

9 polymorphisms are currently known for A33 mRNA and 7 such polymorphisms are present in the non-translation region. Furthermore, one of the remaining 2 polymorphisms is present in the 3rd codon. Hence, this polymorphism is a

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silent mutation that does not undergo amino acid substitution. Furthermore, the other one of the remaining two polymorphisms undergoes amino acid substitution, but is present within the signal sequence. Accordingly, the antibody of the present invention is effective for therapeutic or preventive agents, regardless of A33 polymorphism.

DEMANDES OU BREVETS VOLUMINEUX

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

CECI EST LE TOME __1__ DE __2__

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

JUMBO APPLICATIONS / PATENTS

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

THIS IS VOLUME __1__ OF __2__

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

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CLAIMS:

1. A human antibody which binds to A33 and produced by the hybridoma M10 having accession No. FERM BP-10107.
2. A human antibody which binds to A33 and produced by the
5 hybridoma M96 having accession No. FERM BP-10108.
3. A human antibody which binds to A33 and produced by the hybridoma M165 having accession No. FERM BP-10106.
4. A human antibody which binds to A33 and produced by the hybridoma N26 having accession No. FERM BP-10109.
- 10 5. A human antibody which binds to A33 and produced by the hybridoma Q47 having accession No. FERM BP-10104.
6. A human antibody which binds to A33 and produced by the hybridoma Q54 having accession No. FERM BP-10105.
7. A human antibody which binds to A33 and produced by the
15 hybridoma R5 having accession No. FERM BP-10103.
8. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 23 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 25.
- 20 9. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 27 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 29.
10. A human antibody which binds to A33, in which the amino acid
25 sequence of the variable region of the heavy chain is represented by SEQ ID NO: 31 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 33.

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11. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 35 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 37.
- 5 12. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 39 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 41.
- 10 13. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 43 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 45.
- 15 14. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 47 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 49.
- 20 15. A human antibody which binds to A33, in which the amino acid sequence of the variable region of the heavy chain is represented by SEQ ID NO: 51 and the amino acid sequence of the variable region of the light chain is represented by SEQ ID NO: 53.
16. A preventive or therapeutic agent for colon cancer, which contains the human antibody according to any one of claims 1 to 15.
17. Use of the antibody of any one of claims 1 to 15 for preventing or treating colon cancer.
- 25 18. Hybridoma M10 having accession No. FERM BP-10107.
19. Hybridoma M96 having accession No. FERM BP-10108.
20. Hybridoma M165 having accession No. FERM BP-10106.

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21. Hybridoma N26 having accession No. FERM BP-10109.
 22. Hybridoma Q47 having accession No. FERM BP-10104.
 23. Hybridoma Q54 having accession No. FERM BP-10105.
 24. Hybridoma R5 having accession No. FERM BP-10103.
- 5 25. A method for producing an antibody, which comprises culturing the hybridoma according to any one of claims 18 to 24 and then obtaining the antibody from the culture product.

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

Application number / numéro de demande: JP05 / 16576

Figures: 3A-3B, 4-6

Pages: 9-13

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Documents reçu avec cette demande ne pouvant être balayés
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10^{ème} étage)

Fig. 1A

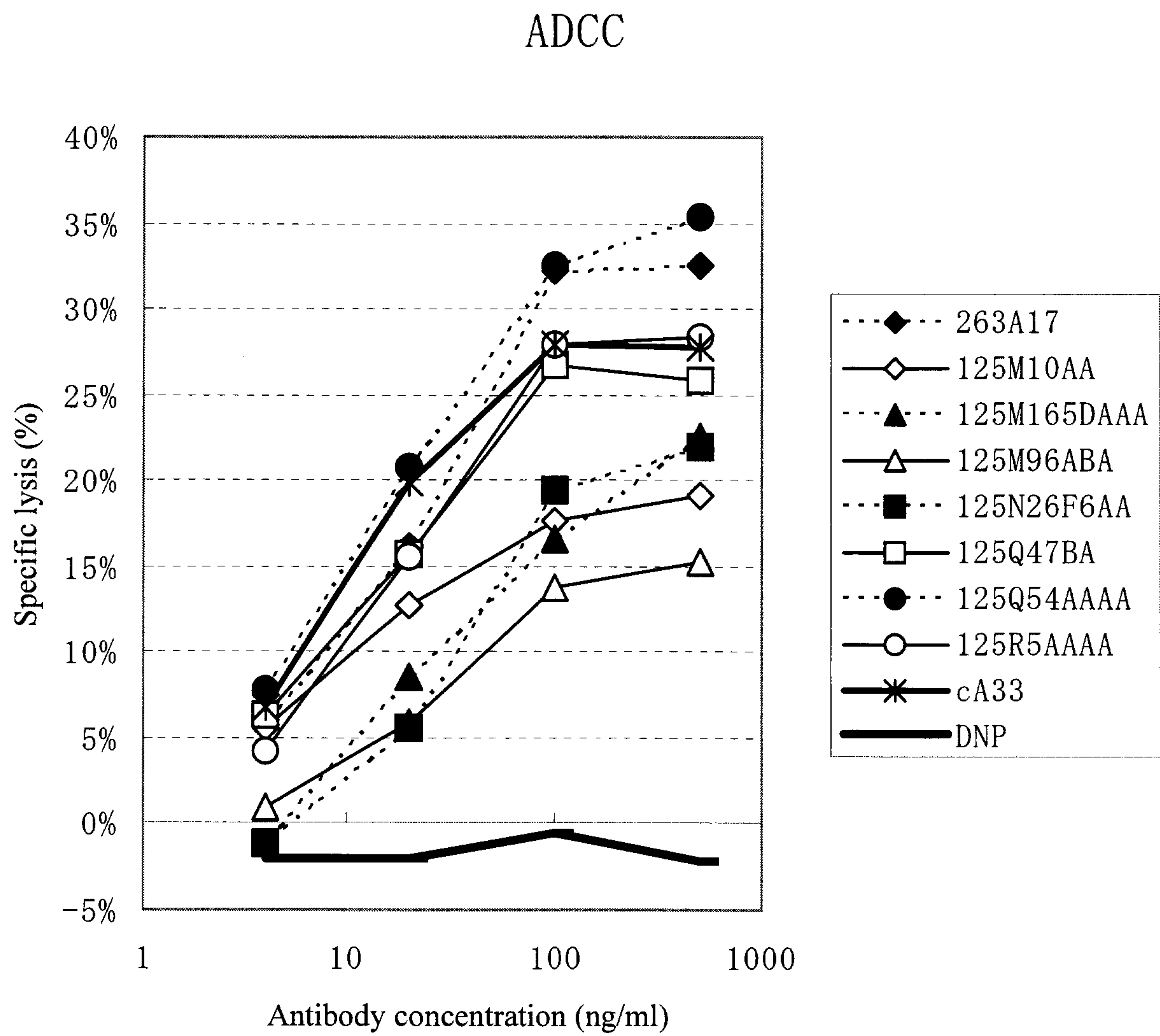


Fig. 1B

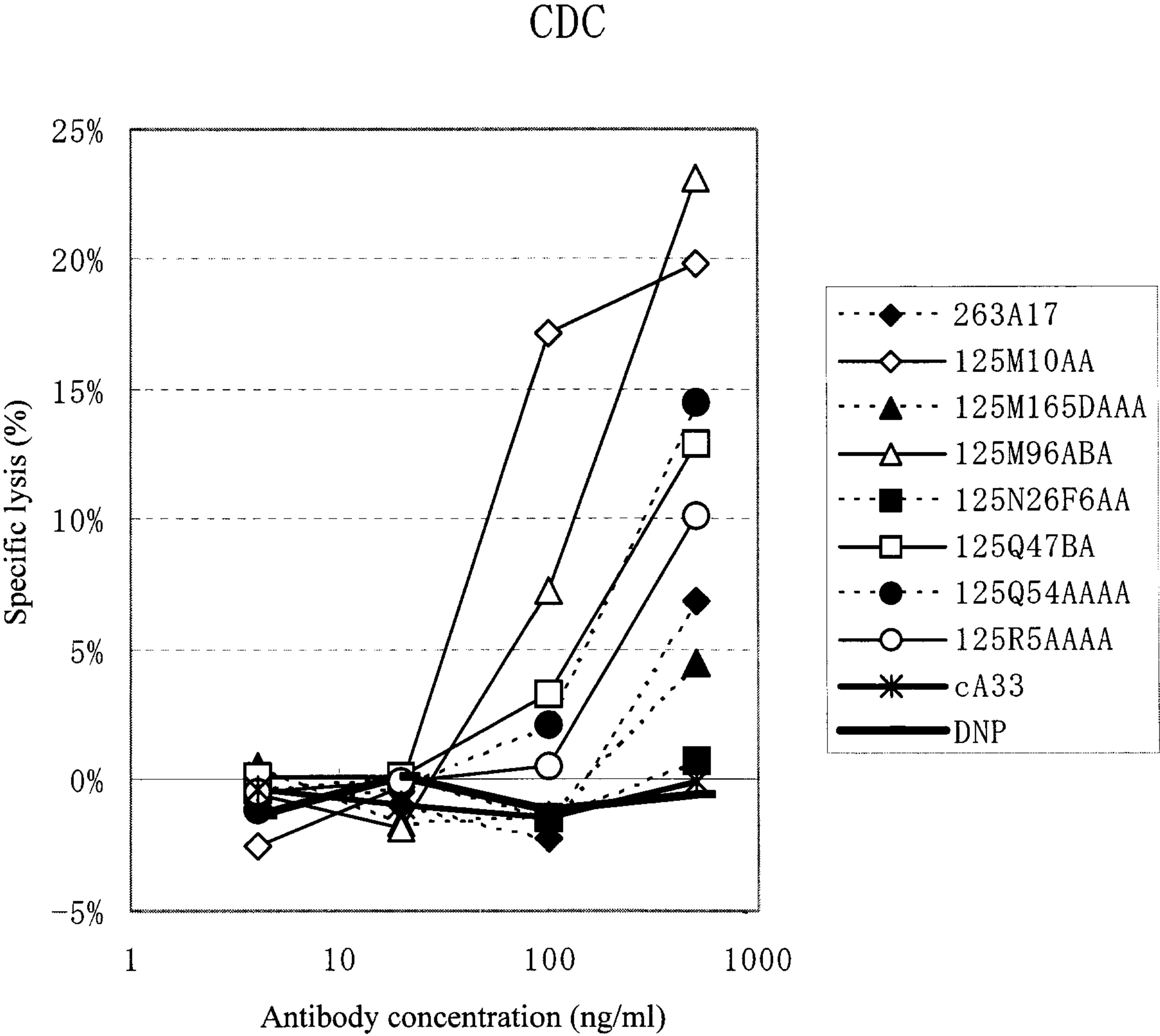


Fig. 1C

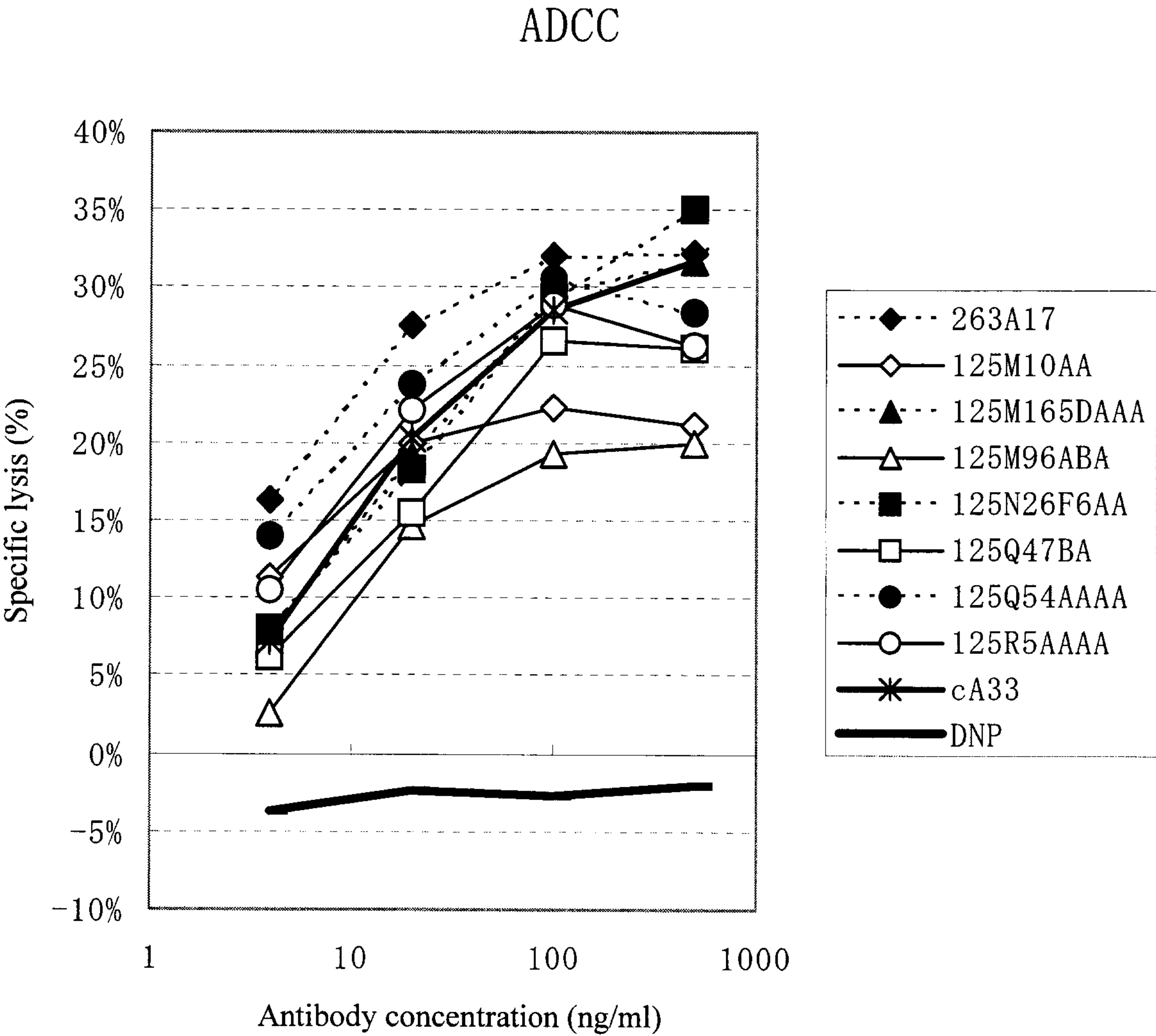


Fig. 1D

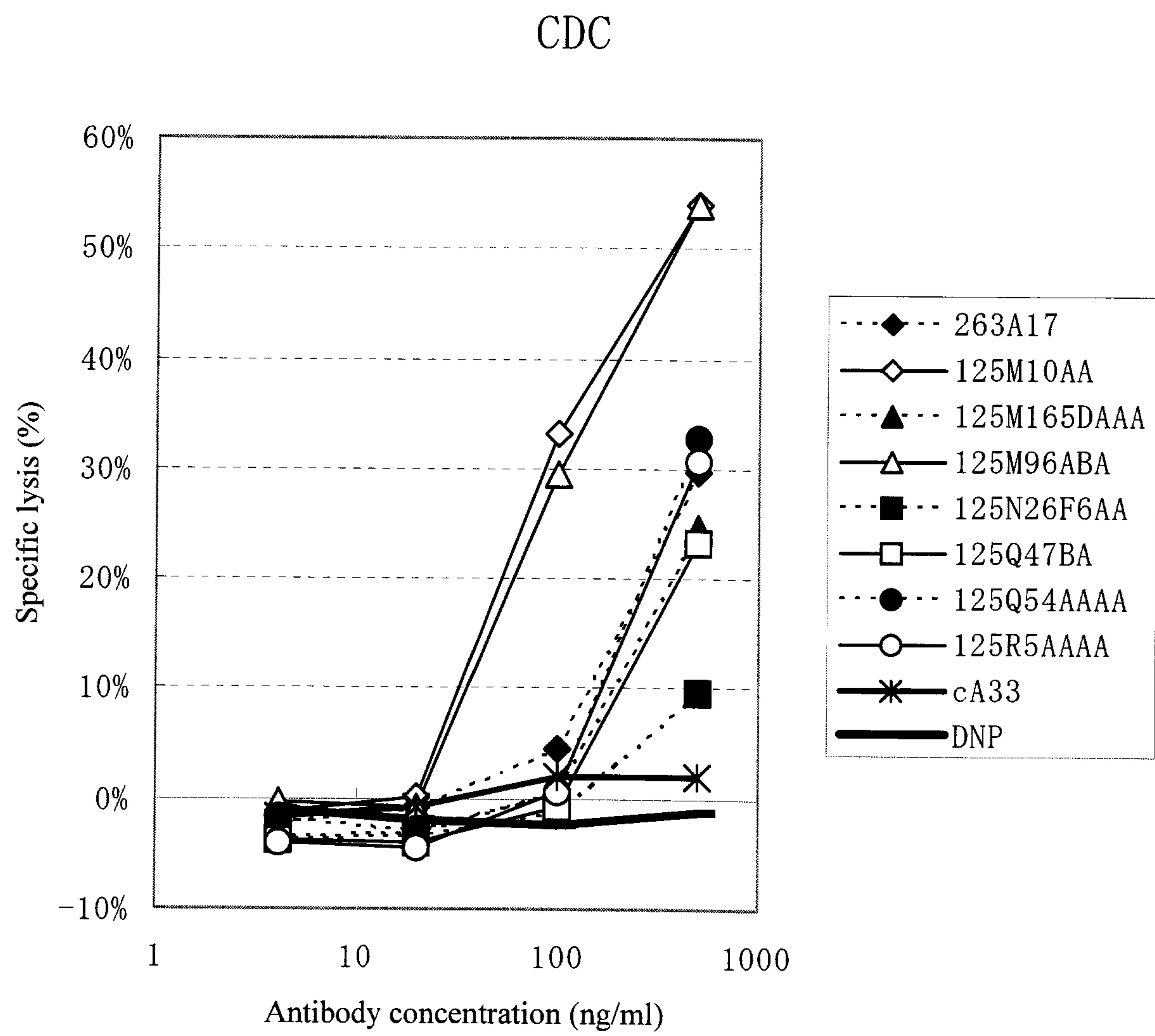


Fig. 2A

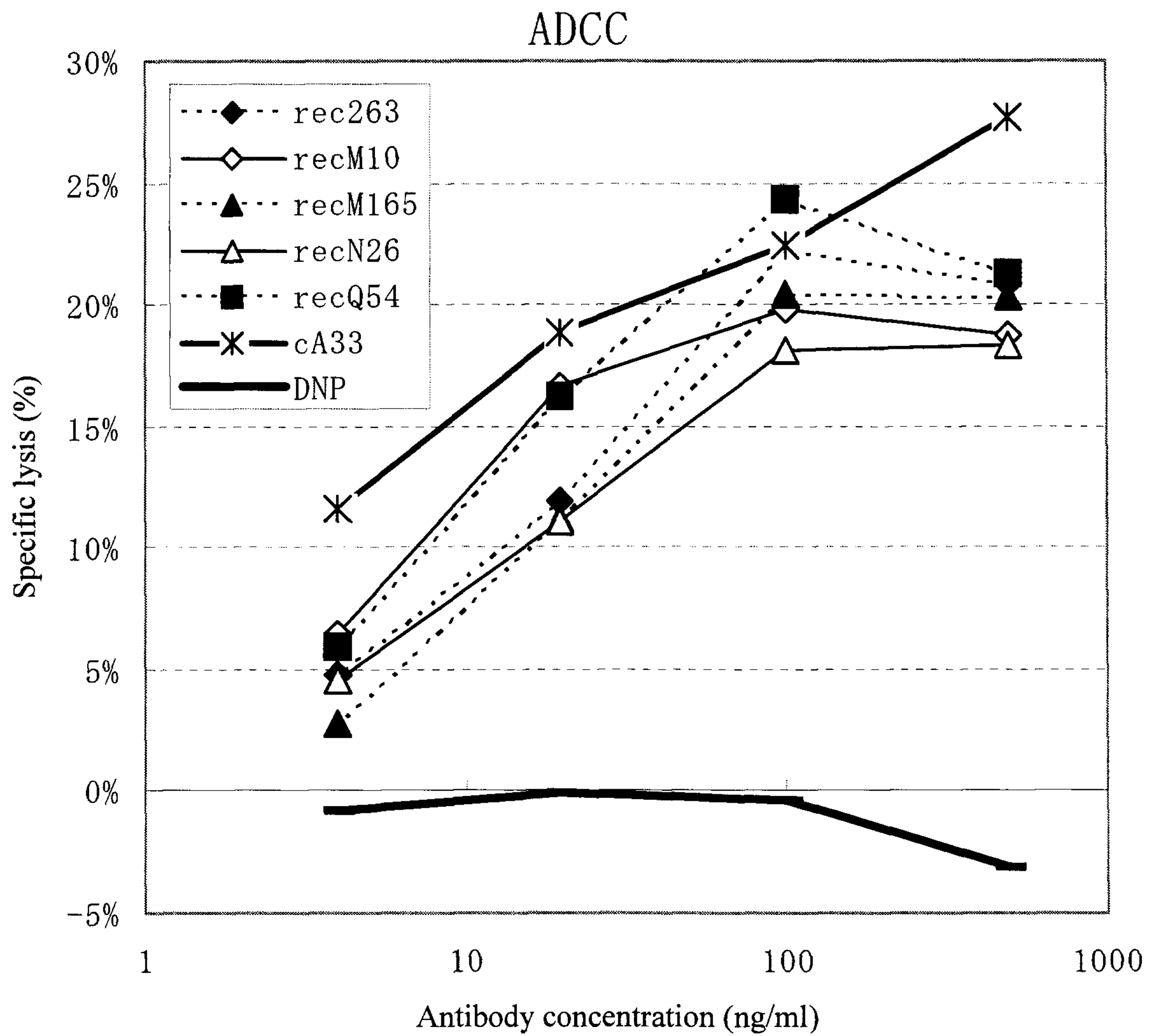


Fig. 2B

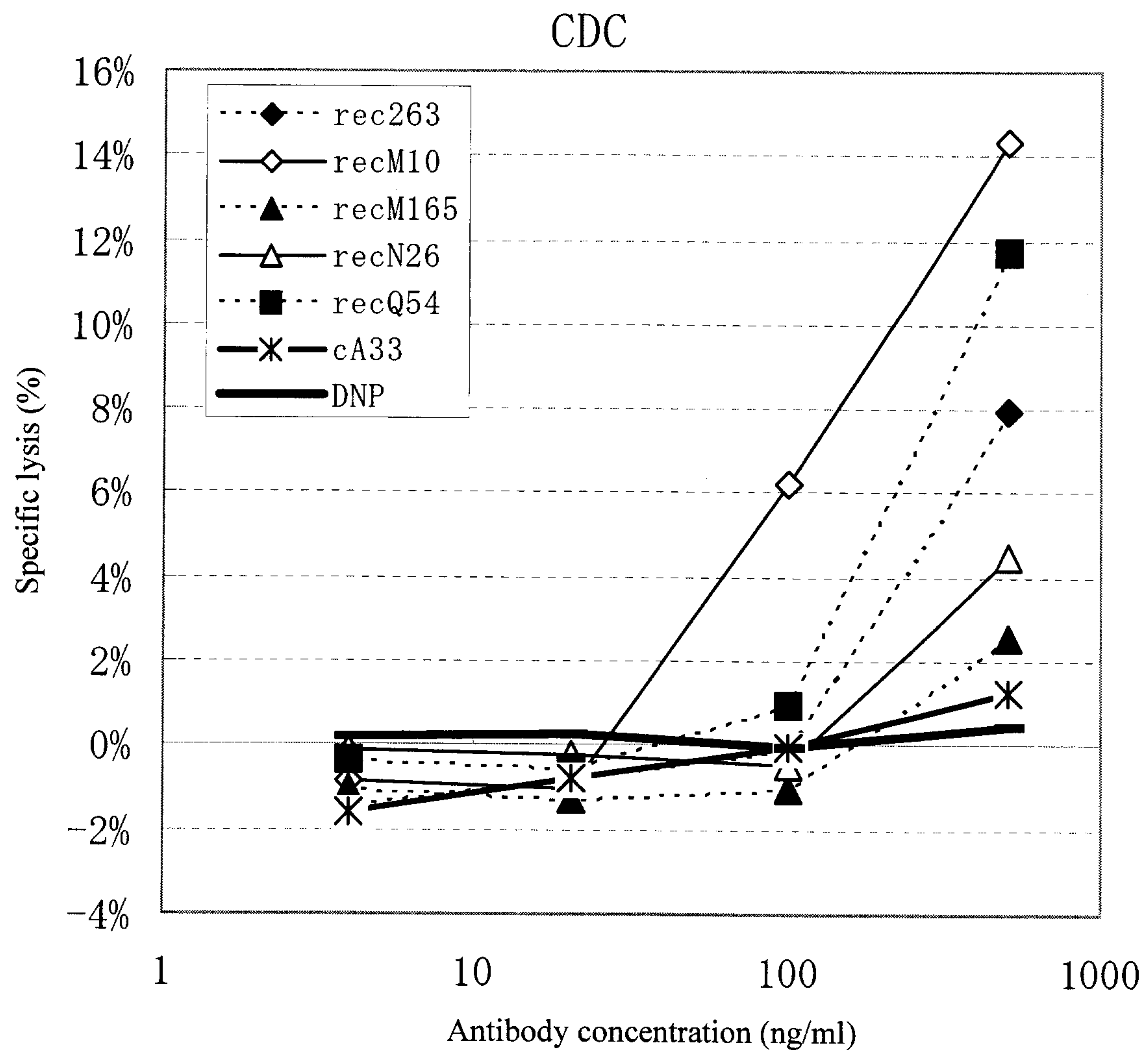


Fig. 2C

ADCC

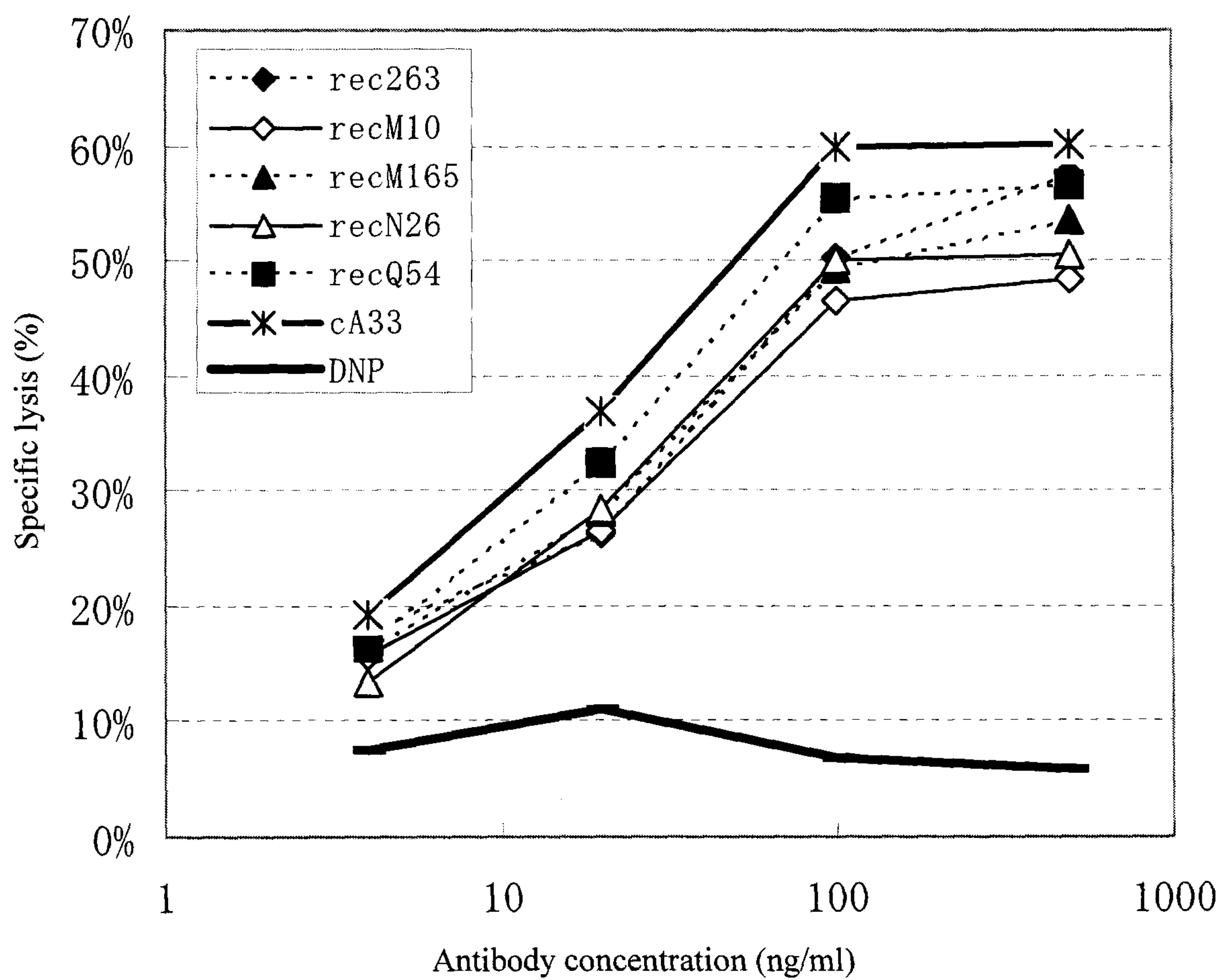


Fig. 2D

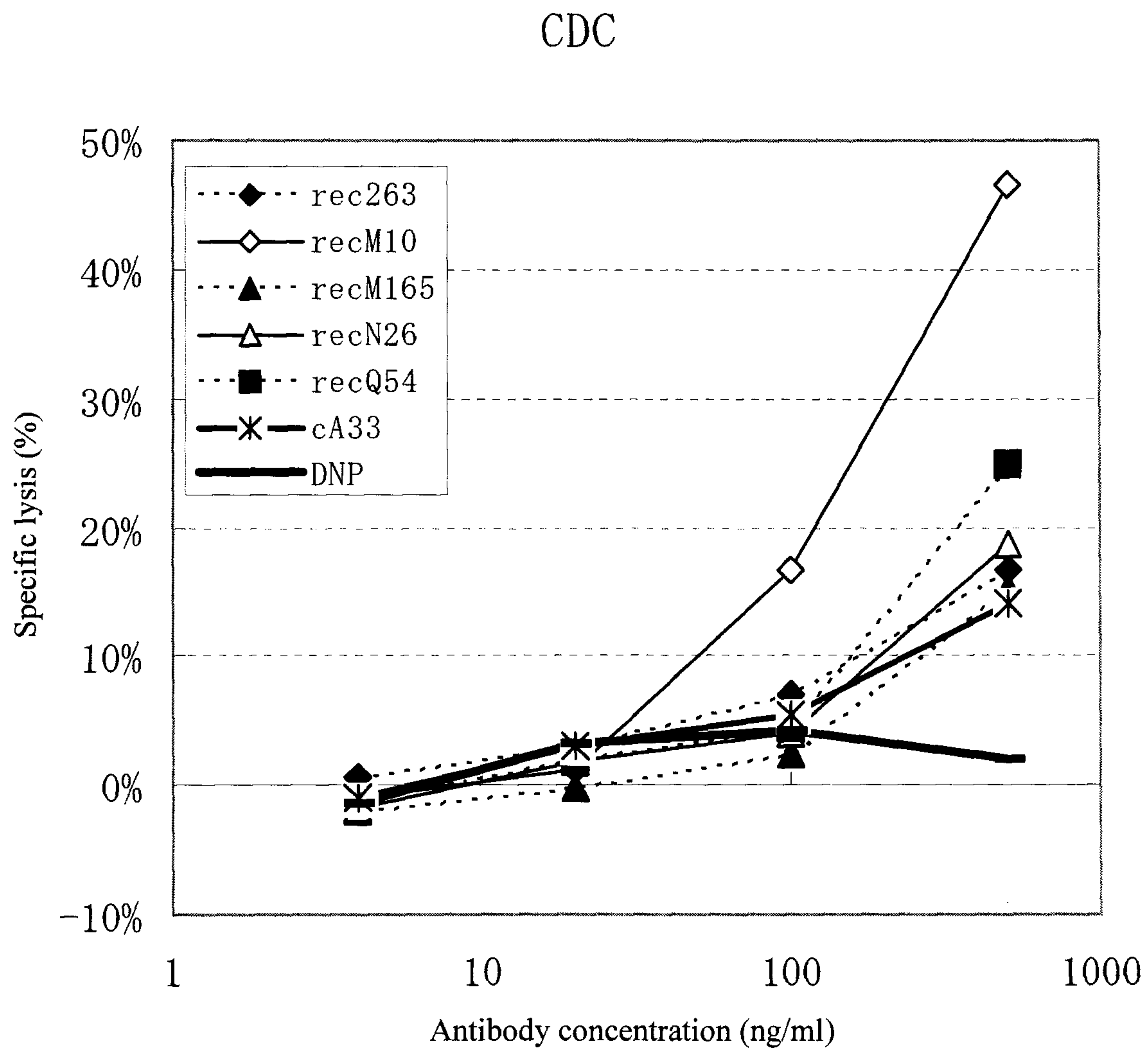


Fig. 7A

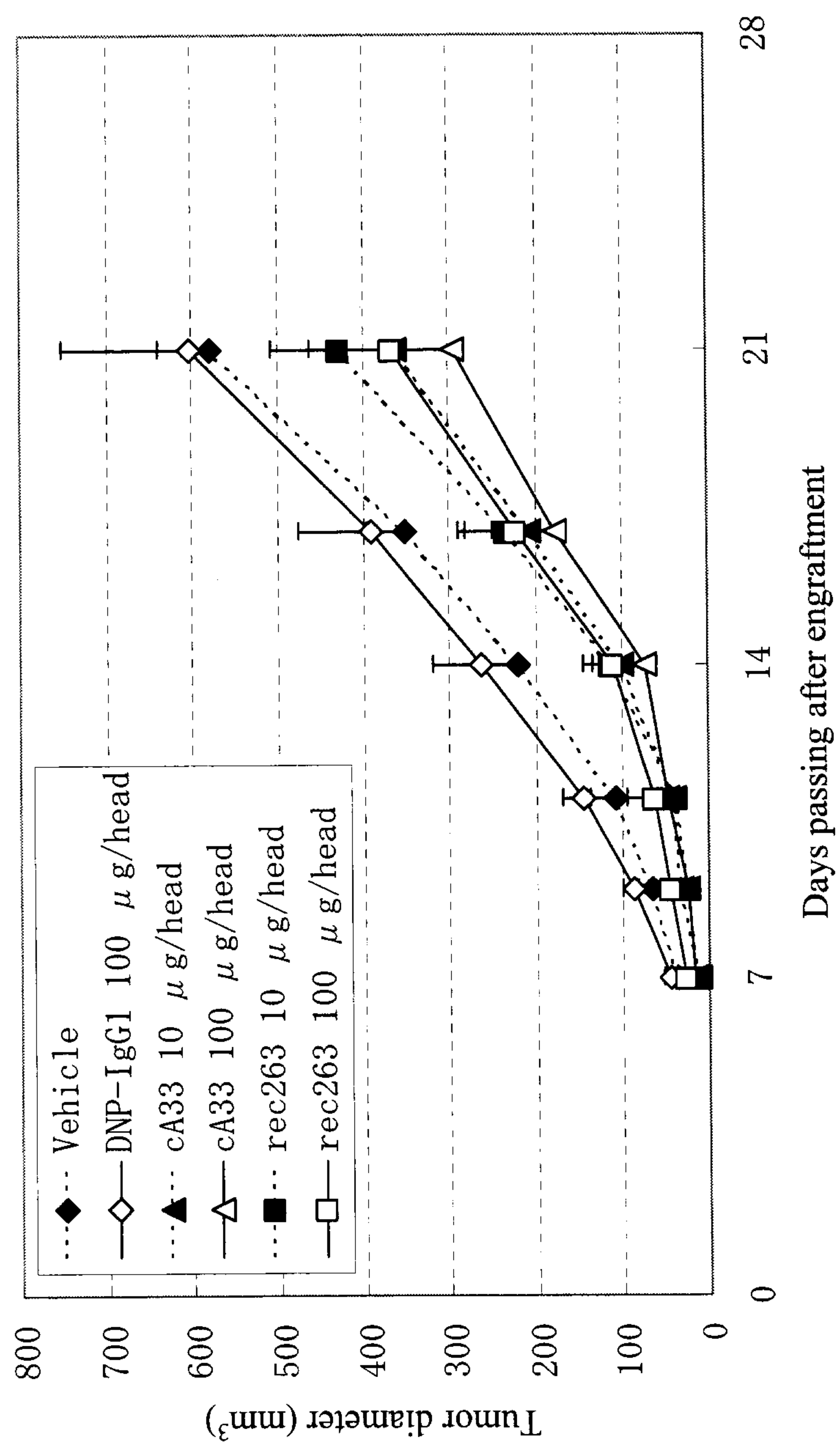


Fig. 7B

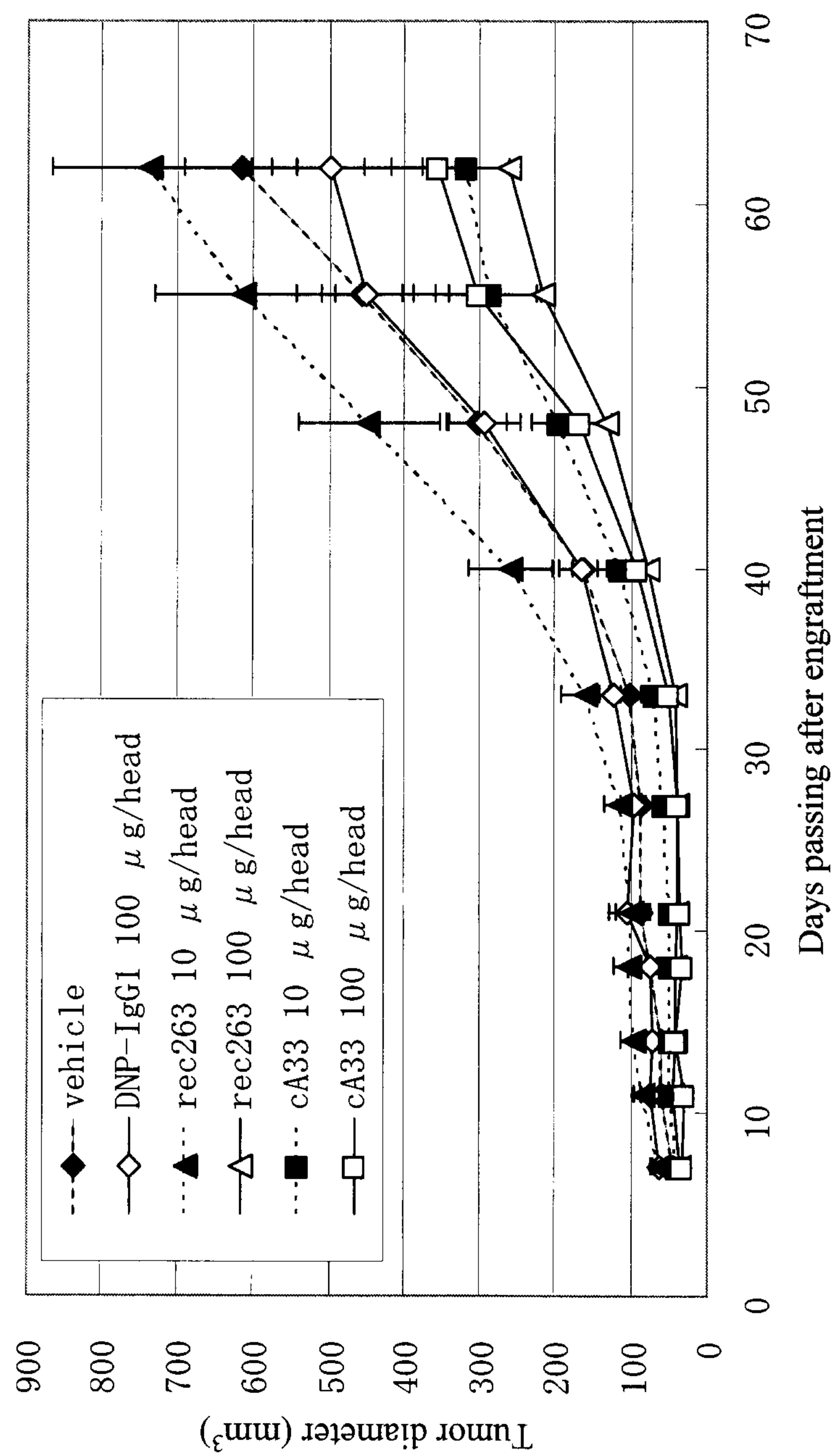
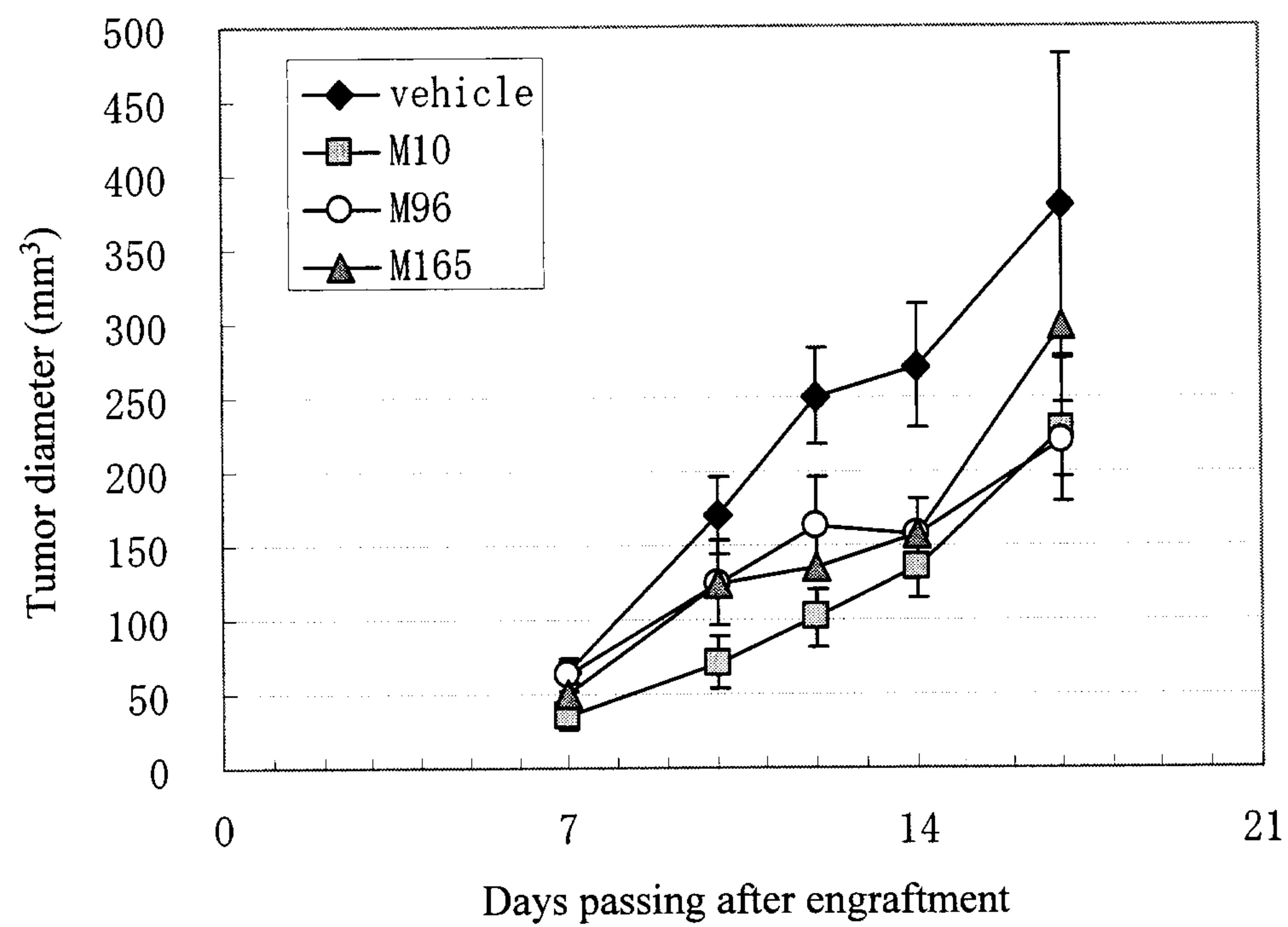
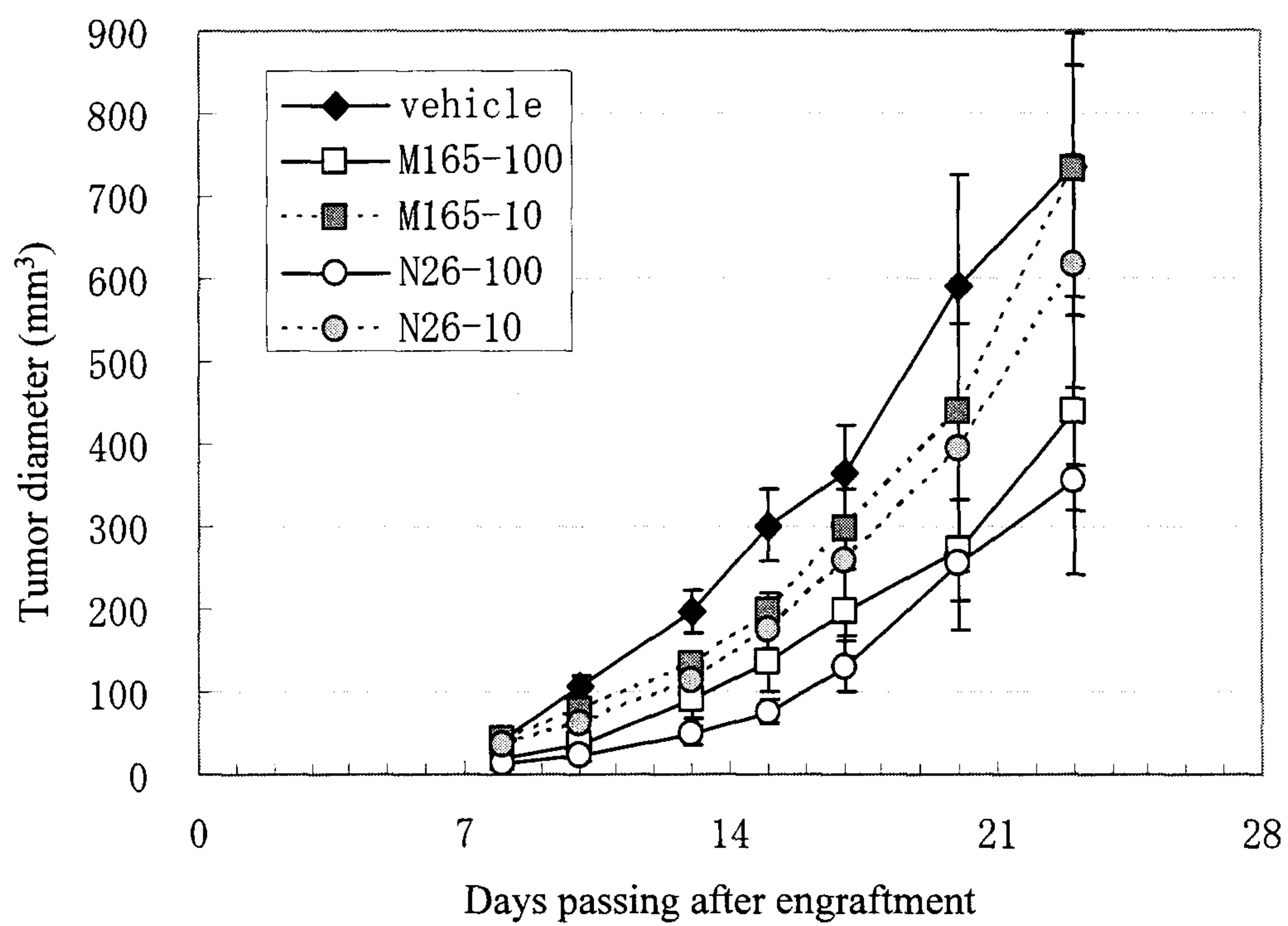


Fig. 7C



M10 : 125M10AA
M96:125M96ABA
M165:125M165DAAA

Fig. 7D



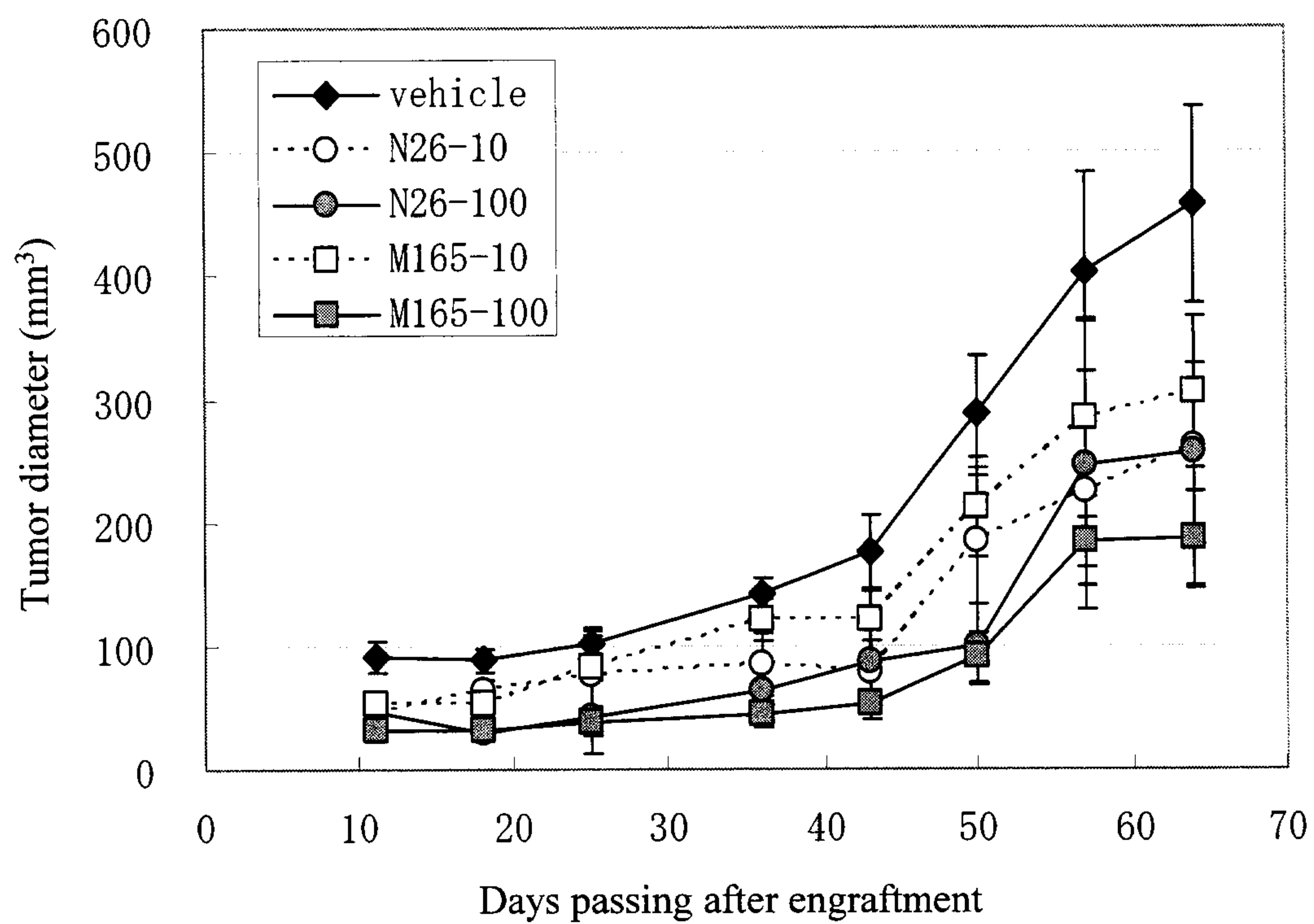
M165-10 : recM165-10 $\mu\text{g}/\text{head}$

M165-100 : recM165-100 $\mu\text{g}/\text{head}$

N26-10 : recN26-10 $\mu\text{g}/\text{head}$

N26-100 : recN26-100 $\mu\text{g}/\text{head}$

Fig. 7E



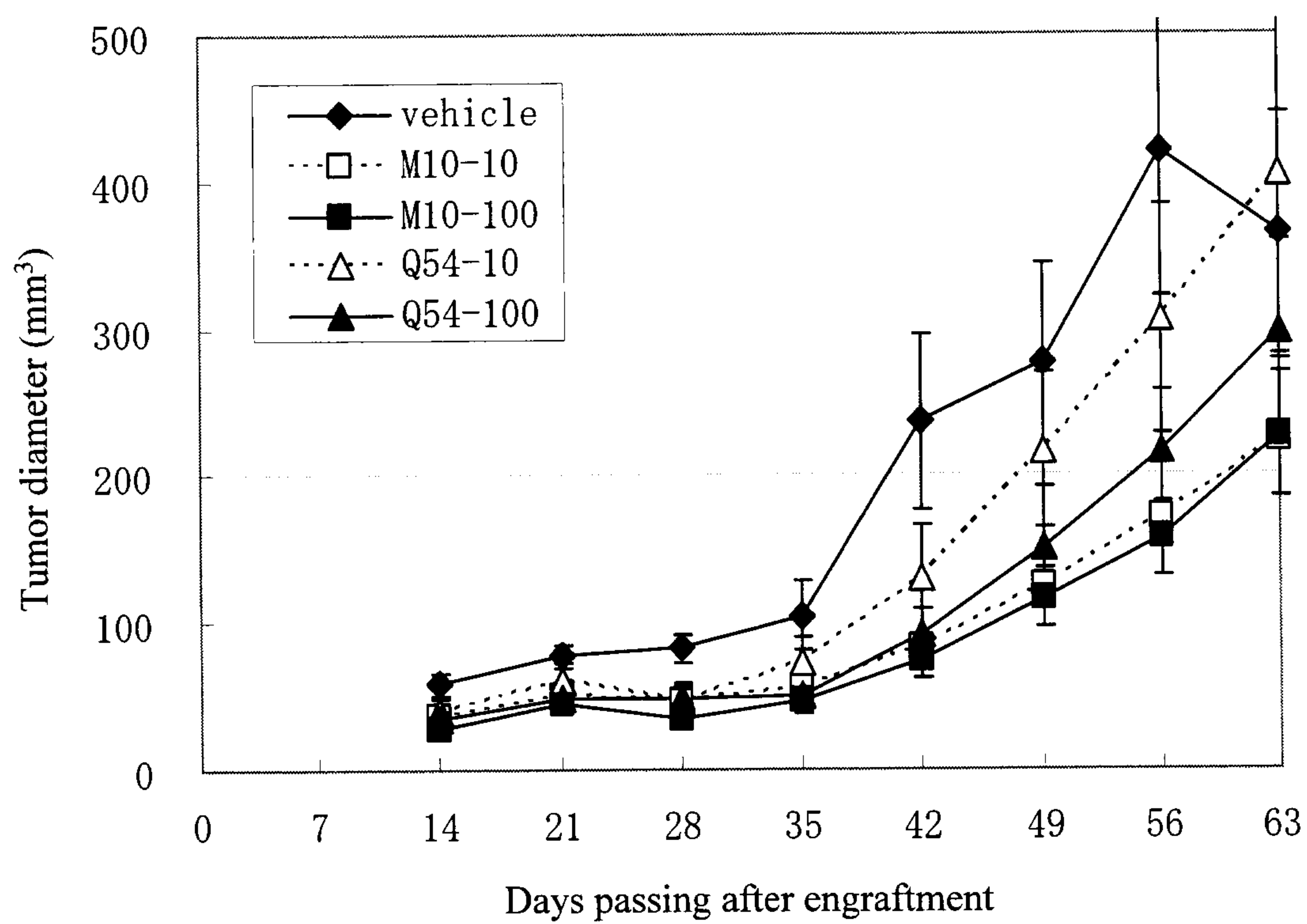
N26-10 : recN26-10 $\mu\text{g}/\text{head}$

N26-100 : recN26-100 $\mu\text{g}/\text{head}$

M165-10 : recM165-10 $\mu\text{g}/\text{head}$

M165-100 : recM165-100 $\mu\text{g}/\text{head}$

Fig. 7F



M10-10 : recM10-10 $\mu\text{g}/\text{head}$

M10-100 : recM10-100 $\mu\text{g}/\text{head}$

Q54-10 : recQ54-10 $\mu\text{g}/\text{head}$

Q54-100 : recQ54-100 $\mu\text{g}/\text{head}$